The Journal of Experimental Biology 212, 934-944
Published by The Company of Biologists 2009
doi:10.1242/jeb.027680

Nervous and humoral control of cardiac performance in the winter flounder (*Pleuronectes americanus*)

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Accepted 20 January 2009

SUMMARY

Previous studies have suggested that flatfish lack adrenergic cardiac innervation and have a limited humoral adrenergic stress response. However, data on neurohormonal control of flatfish cardiac function is scarce, and has never been directly studied *in vivo*. Hence, we (1) injected neural and humoral antagonists into flounder (*Pleuronectes americanus*) *in vivo* to determine the contribution of autonomic innervation and circulating catecholamines to the control of resting cardiac function; (2) measured pre- and post-stress (90 s chase) catecholamine levels in this species; and (3) constructed *in vivo* catecholamine dose–response curves for cardiovascular function based on the results of the second experiment. In addition, we quantified the density (B_{max}) and ligand-binding affinity (K_d) of flounder ventricular cell-surface β-adrenoreceptors, and established whether they were of β_1 or β_2 subtype using pharmacological antagonists. The cholinergic contribution to resting flounder heart rate was comparable to other teleosts (cholinergic tonus 26%). Interestingly, however, bretylium increased heart rate, resulting in a negative resting adrenergic tonus (–11.9%), and we were unable to demonstrate that catecholamines supported cardiac function at rest or at circulating concentrations approximating those following an exhaustive chase (adrenaline, 21 nmol l^{-1}; noradrenaline, 14 nmol l^{-1}). Myocardial B_{max} was very high in the flounder (252.8 fmol mg^{-1} protein), and it appears that flounder ventricular β-adrenoreceptors are predominantly of the β_2 subtype [based on the inability of atenolol to displace [^{3}H]CGP from the β-adrenoreceptors, and the IC_{50} value for ICI 118551 (1.91×10^{-6} mol l^{-1})]. However, the extremely low affinity (K_d 1.02 nmol l^{-1}) for these receptors raises the possibility that the flounder heart is also populated by β_3-adrenoreceptors.

Key words: beta-adrenoreceptors, binding affinity, catecholamines, flatfish, heart, teleost.

INTRODUCTION

Numerous studies have examined the nervous and humoral control of the fish heart. For instance, myxinoid cyclostomes have aneural hearts (Laurent et al., 1983; Axelsson et al., 1990; Taylor et al., 1999) and, in elasmobranchs, nervous control of the heart is mainly attributed to the degree of cholinergic vagal tonus (Taylor et al., 1999; Agnisola et al., 2003). In most teleosts, the heart is innervated by both inhibitory parasympathetic fibres, running in the vagus nerve, and postganglionic sympathetic fibres, reaching the heart directly or *via* the vagus (Donald and Campbell, 1982; Laurent et al., 1983; Farrell and Jones, 1992). Humoral adrenergic stimulation of the heart is also generally important and is mediated by catecholamines released from the chromaffin tissue into the circulatory system (Gamperl et al., 1994c; Reid et al., 1998). Resting levels of plasma catecholamines [adrenaline (A) and noradrenaline (NA)] are usually less than 10 nmol l^{-1}. However, in response to severe stress, the concentration of circulating catecholamines can increase dramatically (i.e. to levels in excess of 300 nmol l^{-1}), promoting metabolic and circulatory adjustments to cope with increasing energetic demands (Randall and Perry, 1992; Gamperl et al., 1994c; Perry and Gilmour, 1999).

Interestingly, the potential for catecholamines to modulate cardiac performance varies with the severity and type of stressor, and among species. For instance, in Atlantic cod (*Gadus morhua*), A and NA concentrations increase by 11.5-fold and 5.6-fold, respectively, at this species' critical swimming speed (U_{crit}) (Butler et al., 1989), whereas no change in plasma catecholamine concentrations was found when cod were swum at only two-thirds of U_{crit} (Axelsson and Nilsson, 1986). Increases in A and NA, of 46-fold and 11.5-fold, respectively, were reported in rainbow trout (*Oncorhynchus mykiss*) after 10 min of chasing (Tang and Boutilier, 1988), while chasing till exhaustion can raise A and NA by as much as 92-fold and 20-fold, respectively (Perry et al., 1996). Circulating catecholamine concentrations also differ greatly between fish species and, in general, species with a more active lifestyle exhibit greater increases in plasma catecholamine levels when exposed to stressors as compared with benthic/sluggish species. Thus, it is not surprising that maximum A and NA levels in the sea raven (*Hemitripterus americanus*; a North Atlantic benthic species) after 1 min of air exposure followed by 1 min of chasing are only ~8 nmol l^{-1} (Vijayan and Moon, 1994), and that A and NA levels are only ~30 and 37 nmol l^{-1}, respectively, in the starry flounder [*Platichthys stellatus* (Milligan and Wood, 1987)] after 10 min of chasing. By contrast, the A concentration in rainbow trout can be as high as 275 nmol l^{-1} after being chased to exhaustion (Perry et al., 1996), and circulating A concentrations of 565 nmol l^{-1} have been found in Atlantic cod after exposure to severe acute hypoxia (L. H. Petersen and A.K.G., unpublished data).

Flatfishes (order Pleuronectiformes) are unique because, in contrast to most teleosts, electrophysiological and histochemical studies suggest that adrenergic cardiac innervation is absent (Cobb and Santer, 1973; Donald and Campbell, 1982; Ask, 1983). Furthermore, flounder appear to have a limited humoral adrenergic stress response (Milligan and Wood, 1987), a characteristic probably related to their benthic and inactive lifestyle (Pereira et al., 1999). Given the lack of cardiac sympathetic innervation and the low post-

stress circulating catecholamine levels reported, and that neurohormonal control of flatfish cardiac function has never been directly studied, it is not clear how this taxa regulates cardiac function. Thus, the purpose of this study was to determine how *in vivo* cardiovascular function is regulated in the winter flounder (*Pleuronectes americanus* Walbaum 1792) by nervous and humoral mechanisms. To accomplish this a number of experiments were undertaken: (1) a series of neural and humoral antagonists were used to determine the contribution of autonomic innervation and circulating catecholamines to the control of *in vivo* heart function; (2) maximal post-stress circulating catecholamines were measured in the flounder following two different stressors (a 60 s net stress and a 90 s chase); (3) *in vivo* dose–response curves for catecholamines (A and NA) were produced to examine the ability of circulating catecholamines to stimulate the winter flounder heart; and (4) flounder ventricular β-adrenoreceptors were typed and quantified, to better understand how β-adrenoreceptors relate to, and mediate, the effects of catecholamines on the heart of this species.

MATERIALS AND METHODS

Animals

Ethical approval for the following studies was obtained from the Animal Care Committee at Memorial University (protocol # 05-02-KG). Winter flounder were caught by divers using a hand net in Conception Bay (Newfoundland, Canada) at a depth of 4–6 m and transported to the Ocean Sciences Centre (OSC, Memorial University of Newfoundland). Flounder were acclimated to 8±1°C for at least 4 weeks prior to experimentation in 1200 l rectangular fibreglass tanks supplied with aerated seawater and natural photoperiod. Fish were fed three times a week with commercial pellets, but were fasted for 24 h prior to surgery.

In vivo experiments

Surgery

Fish were anaesthetized (average mass 0.46±0.13 kg) in seawater containing methane sulfonic acid of *m*-aminobenzoate (MS-222; 0.25 g l^{-1}), and then transferred to a surgical table, where their gills were irrigated with chilled (~4°C) and oxygenated seawater containing 0.1 g l^{-1} MS-222.

Implantation of flow probe

Implantation of the blood flow probe was performed as previously described by Crocker et al. (Crocker et al., 2000) for white sturgeon, with some modifications. In this procedure, the gills and operculum were retracted using umbilical tape (Baxter Healthcare Corporation, Deerfield, IL, USA) which was passed from a hole behind the fourth gill arch into the opercular cavity, the ventral aorta was exposed through a small incision (~0.5 cm) in the isthmus without disrupting the pericardium, and a flow probe (1.5RB, Transonic Systems; Ithaca, NY, USA) was placed loosely around the vessel. Finally, after verifying the quality of the cardiac output signal, the probe lead was sutured to the eyed side of the fish using 3–0 silk (American Cyanamid Company, Pearl River, NY, USA) at three locations.

Cannulation of caudal artery

Cannulation of the caudal artery for the measurement of dorsal aortic pressure was performed as previously described by Cech and Rowell (Cech and Rowell, 1976) with modifications. Briefly, a 2 cm long incision was made, just below the lateral line at about one third of the animal's length from the tail. The skin and underlying muscle tissue were then retracted to expose the caudal artery which lies between the haemal arches, and a heparinized cannula (PE 50, Clay Adams, Parsippany, NJ, USA; 80 cm long, volume 0.2 ml) with indwelling 14 gauge piano wire was inserted into the vessel. Finally, after removing the indwelling wire, and pushing the cannula approximately 8 cm anteriorly into the artery, the incision was closed with a continuous suture, and the cannula was filled with heparinized saline ⟨181.3 mmol l^{-1} NaCl, 5.0 mmol l^{-1} KCl, 2.30 mmol l^{-1} $CaCl_2 \cdot 2H_2O$, 1.99 mmol l^{-1} $MgSO_4 \cdot 6H_2O$, 2.58 TES acid {*N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid} and 7.33 mmol l^{-1} sodium TES base {*N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid sodium salt} with 100 i.u. ml^{-1} heparin⟩, and sutured to the fish's dorsal surface at two locations. There was minimal bleeding during cannula implantation, and the cannula was flushed regularly with heparinized saline to prevent clot formation.

Recovery from anaesthesia was initiated after surgery by artificially ventilating the fish with aerated, anaesthetic-free water. Once ventilatory activity had returned, the fish were placed into an opaque 451 cooler supplied with aerated 8°C seawater and filled with ~5 cm of sand. The flounder were then allowed to recover for at least 24 h prior to experimentation.

Experimental procedures

Neural control of cardiac function

Following the recovery period, cardiac output ($\dot{Q}$), dorsal aortic pressure (P_{DA}) and heart rate (f_H) were recorded for 1 h. After this initial recording period, a series of drugs were injected every 1 h 30 min in the following order: 1.2 mg kg^{-1} atropine sulphate (muscarinic receptor antagonist); 10 mg kg^{-1} bretylium tosylate (adrenergic nerve blocker), 213 μg kg^{-1} (RS)-atenolol (β_1-adrenoreceptor antagonist) and 250 μg kg^{-1} ICI 118551 hydrochloride (β_2-adrenoreceptor antagonist). These drug concentrations were selected based on previous fish studies (Smith et al., 1985; Altimiras et al., 1997; van Heeswijk et al., 2005), and all drugs were injected slowly (over approx. 15 s) through the caudal artery in a concentrated form using a 1 ml kg^{-1} carrier volume of saline. This initial injection was followed by an injection of enough saline (~0.3 ml) to ensure complete delivery of the drug into the animal.

Chasing and net stress

In order to determine maximal post-stress circulating catecholamines and haematocrit levels, two groups of flounder were used in which no surgical procedure was performed. One group of flounder (*N*=8) was held in a net for 60 s, while another group (*N*=8) was chased to exhaustion prior to sampling. In the chasing procedure, each fish was caught individually from their holding tanks using a net and chased immediately in a rectangular tank (1 m×1 m×0.5 m) for 90 s using a small wooden prod. Immediately after these procedures were finished, blood samples were taken from the flounder (0.6–0.9 ml) for the measurement of post-stress haematocrit and plasma catecholamine (A and NA) levels by caudal puncture.

For the measurement of catecholamines, the blood was immediately placed into a chilled 1.5 ml Eppendorf centrifuge tube, and centrifuged at 10,000 ***g*** for 30 s to obtain plasma. The plasma was then pipetted into a 1.0 ml cryovial containing glutathione and EDTA (5 μl of 0.2 mol l^{-1} glutathione and 5 μl of 0.2 mol l^{-1} EDTA per 100 μl of plasma), and immediately frozen in liquid N_2. All plasma samples were then stored at –80°C until analysis.

Catecholamine dose–response curves

To assess the sensitivity of the flounder cardiovascular system to circulating catecholamines, dose–response curves for A and NA were generated from *in vivo* measurements. As described above,

fish (N=7) were implanted with a ventral aortic flow probe and a caudal artery cannula, and then allowed to recover for approx. 24 h. After recovery, $\dot{Q}$, P_{DA} and f_H were recorded for 1 h, and then each fish was given a series of five 1 ml kg^{-1} saline injections. Each saline injection was separated by 1 h to assess the potential effects of haemodilution on the flounder's cardiovascular function, and thus discern sham injection effects from drug effects (see below). Subsequent to the saline injections, the flounder were allowed to recover for 18 h, after which they were injected with 0.1 and 0.05 (dose 1), 0.15 and 0.075 (dose 2), 0.2 and 0.1 (dose 3), 0.3 and 0.15 (dose 4), and finally 0.4 and 0.2 μg kg^{-1} (dose 5) of A and NA, respectively. These injections were given at 1 h intervals (an inter-injection period that allowed cardiovascular variables to return to resting values), and all doses of A and NA were injected slowly (over approx. 15 s) through the caudal artery in a concentrated form using a 1 ml kg^{-1} carrier volume of saline. These concentrations were selected based on the maximum concentrations of A (21 nmol l^{-1}) and NA (14 nmol l^{-1}) found in winter flounder after chasing, and the relationship between injected dose and plasma A levels established by Gamperl et al. (Gamperl et al., 1994b) for rainbow trout at similar temperatures. Cardiovascular variables and P_{DA} were measured for 10 min before and 45 min after each injection. Blood samples were also taken prior to the first injection (0.5 ml) and 2 min after the last injection (0.8 ml) for the determination of plasma A and NA concentrations induced by the injection of 0.4 and 0.2 μg kg^{-1} (dose 5) of A and NA, respectively. Immediately upon collection, blood samples were processed using the same protocol as in the chasing and net stress experiments.

Data analysis

Instrumentation for cardiovascular measurements

Dorsal aortic pressure (P_{DA}, in kPa) was measured using a Gould Statham pressure transducer (Model P23 ID, Oxnard, CA, USA) that was calibrated daily against a static water column, where zero pressure (0 kPa) was set equal to the water level in the experimental chamber. Cardiac output ($\dot{Q}$; ml min^{-1} kg^{-1}) was monitored by connecting the flow probe lead to a small animal blood flow meter (Model T206, Transonic® Systems, Ithaca, NY, USA). Both pressure and flow signals were amplified and filtered using a Model MP100A-CE data acquisition system (BIOPAC Systems, Santa Barbara, CA, USA), and analyzed and stored using Acq*Knowledge* Software (BIOPAC Systems, Santa Barbara, CA, USA) installed on a 300 MHz Toshiba laptop computer.

Calculation of cardiovascular parameters

Cardiovascular function was continuously monitored throughout the 'neural control of cardiac function' and 'dose–response curve' experiments by measuring $\dot{Q}$ and P_{DA}. Heart rate (f_H; beats min^{-1}) was calculated by measuring the number of systolic peaks during 20–30 s intervals. Mass specific stroke volume (V_S; ml kg^{-1}), was calculated as: V_S=cardiac output (ml min^{-1} kg^{-1})/heart rate (beats min^{-1}). Systemic vascular resistance (R_{sys}; kPa ml^{-1} kg min) was calculated as: dorsal aortic pressure (kPa)/cardiac output (ml min^{-1} kg^{-1}).

The 'intrinsic' (after the administration of all drugs) f_H (f_{Hint}; beats min^{-1}), cholinergic tonus (%f_{Hch}; %) and adrenergic tonus (%f_{Had}; %) were calculated for the winter flounder heart as described by Axelsson (Axelsson, 1988):

$$\%f_{Hch} = [(f_H \text{ after atropine} - f_H \text{ before atropine}) / f_{Hint}] \times 100 , \quad (1)$$

$$\%f_{Had} = [(f_H \text{ after atropine} - f_{Hint}) / f_{Hint}] \times 100 . \quad (2)$$

Catecholamine analysis

The plasma catecholamines, adrenaline (A) and noradrenaline (NA), were measured using high performance liquid chromatography (HPLC; Bioanalytical Systems, West Lafayette, IN, USA) with electrochemical detection (LC Epsilon® detector, model E5, Bioanalytical Systems) after extraction with alumina (Woodward, 1982). For amine separation, a reverse phase column (ODS, 3.0 mm i.d.×10 cm long, 3 μm pore size, model MF 8954) was used in conjunction with an aqueous mobile phase (containing per litre: 7.088 g monochloroacetic acid, 186.1 mg $Na_2EDTA{\cdot}2H_2O$, 15 ml acetonitrile and 232.3 mg sodium octyl sulphate, pH 3.00–3.05), pumped (PM 80, BAS) at a flow rate of 1 ml min^{-1}. DHBA (3,4-dihydroxybenzylamine) was used as an internal standard for all plasma samples and catecholamine standards. The recovery of DHBA (3,4-dihydroxybenzylamine) from the alumina was in the order of 50–80%, and was used to determine individual plasma catecholamine concentrations. The output from the detector was collected and analysed using a computer running ChromGraph Control and ChromGraph Report version 2.30 software (Bioanalytical Systems).

Chemicals

Components of the saline were purchased from Fisher Scientific (Fairlawn, NJ, USA), with the exception of TES salt, which was purchased from Sigma Chemical Co. (St Louis, MO, USA). MS-222 was purchased from Syndel Laboratories (Vancouver, BC, Canada). Atropine sulphate salt, bretylium tosylate, (RS)-atenolol, ICI 118551 hydrochloride, (±) timolol, adrenaline bitartrate, noradrenaline bitartrate and all chemicals used in catecholamine extraction and analysis were also purchased from Sigma Chemical Company.

In vitro experiments

Cardiac β-adrenoreceptors

The punch-technique for the measurement of ventricular β-adrenoreceptors was performed as previously described by Gamperl et al. (Gamperl et al., 1994a) for rainbow trout. Flounder (average mass 0.55±0.03 kg) were killed by a blow to the head, and the heart was quickly removed and allowed to beat for approximately 1 min in cold (0–2°C) saline to remove erythrocytes from the ventricular lumen. The ventricle was then quickly removed, cut in half and frozen (in ~2 min) onto the tissue chopper (McIlwain tissue chopper, Brinkmann, Mississauga, ON, Canada) stage before being sliced into 400 μm thick cross sections. The tissue slices were then placed in a Sylgard-coated tissue culture dish filled with ice-chilled saline, and ventricular tissue punches (2 mm diameter, 0.9–1.25 mg) were taken from the slices using a sample corer (Fine Science Tools, Vancouver, BC, Canada). Finally, individual punches were placed in separate wells of a tissue culture plate (Becton Dickinson and Company, Franklin Lakes, NJ, USA), with each well containing 500 μl of saline.

β-Adrenergic receptor density and affinity were measured by incubating the punches in varying concentrations (0.05–2 nmol l^{-1}) of the hydrophilic β-antagonist [^{3}H]CGP-12177 (specific activity 37 Ci mmol^{-1}; Amersham Biosciences, Amersham, Bucks, UK) for 2 h. Non-specific binding (NSB) was measured in the presence of 10^{-5} mol l^{-1} timolol (β-adrenoreceptor antagonist), and was subtracted from total counts to determine specific binding. To express B_{max} (i.e. the density of β-adrenoreceptors on the cell surface) as fmol μg^{-1} protein, the protein content of representative punches was determined using a Bradford protein assay (Coomassie Plus, Pierce Biotechnology, Rokford, IL, USA) with

bovine serum albumin (ICN Biomedical, Aurora, OH, USA) as a standard. During the incubation period all tissue culture plates were kept on ice (i.e. at 0°C) and covered with aluminium foil to prevent the photodegradation of [^{3}H]CGP and its competitors. Following incubation, aliquots of buffer were removed to determine the free concentration of [^{3}H]CGP-12177, and the tissue punches were washed twice in ice-chilled saline and placed into 7 ml scintillation vials containing 5 ml of Ecolume (ICN Canada, Montreal, QC, Canada). All scintillation vials were shaken and allowed to sit for at least 18 h prior to counting. Radioactivity was quantified using a liquid scintillation counter (Packard Tri-Carb 2100TR; Meriden, CT, USA). For the determination of [^{3}H]CGP-binding specificity (i.e. the construction of competition curves), punches were incubated with 1.5 nmol l^{-1} [^{3}H]CGP and with various concentrations (10^{-4}–10^{-9} mol l^{-1}) of atenolol (β_1-adrenoreceptor antagonist) or ICI 118551 (β_2-adrenoreceptor antagonist).

The small size of the winter flounder ventricle required the pooling of tissue punches from two individuals to construct each binding curve. To assess the specific binding of [^{3}H]CGP-12177 to ventricular β-adrenoreceptors, a total of eight binding curves were obtained and each ligand concentration had three (only at 0.05 nmol l^{-1}) to six replicates. For both atenolol and ICI 118551 competition curves, a total of six curves were constructed and each competitor concentration had five to six replicates.

Statistical analyses

The reported variables are expressed as means ± standard error of the mean (s.e.m.). Univariate general linear models were used to test for significant differences between the saline and drug injection groups and between the saline and catecholamine injection groups. When a significant difference between groups was detected, one-way ANOVAs were performed to test for differences between values obtained with saline and corresponding drug/catecholamine injections, and contrasts were performed to test for significant differences within groups. Differences between circulating catecholamine concentrations measured at rest, and after net stress, chasing and catecholamine dose 5 were assessed using one-way ANOVAs. Saturation-binding curves for CGP were analysed, and values for K_d ([^{3}H]CGP dissociation constant; in nmol l^{-1}) and B_{max} (fmol μg^{-1} protein) were determined using Scatchard plots as described by Zivin and Waud (Zivin and Waud, 1982). Competition curves were fitted, and IC_{50} values (i.e. the concentration of ligand that reduced [^{3}H]CGP binding by 50%) were determined using SigmaPlot Software for Windows version 10.0 (Systat Software, Chicago, IL, USA). All statistical analyses were performed using SPSS software for Windows version 15.0 (SPSS, Chicago, IL, USA) and $P<0.05$ was used as the level of statistical significance.

RESULTS

In vivo experiments

Neural control of cardiac function

Cardiovascular parameters reached stable values at approx. 20 min after the injection of drugs, and the repeated injection of saline had only minor effects on cardiovascular parameters measured at 1 h post-injection, indicating that the observed changes in cardiac function, P_{DA} and R_{sys} were directly related to the effects of the various drugs (Figs 1 and 2). A significant positive chronotropic effect was induced by the administration of atropine, with f_H increasing from 25.3 to 36 beats min^{-1}. This increase in f_H was coincident with a significant decrease in V_S (from 0.41 to 0.3 ml kg^{-1}), and thus $\dot{Q}$ remained constant at approx. 10 ml min^{-1} kg^{-1} following atropine injection (Fig. 2). Interestingly, bretylium administration did not result in a decrease in f_H, but a further increase to 40 beats min^{-1}, and had no significant effects on either V_S or $\dot{Q}$. Finally, none of the measured cardiac parameters were affected by the injection of the β_1- and β_2-adrenoreceptor blockers (atenolol and ICI 118551, respectively). Based on the changes in f_H following administration of the various drugs, intrinsic f_H, and cholinergic and adrenergic nervous tones were calculated to be 40.9 beats min^{-1}, 26.1% and –11.9%, respectively (Table 1).

P_{DA} increased from 3.03 to 3.37 kPa (by 11%) following atropine injection and, because $\dot{Q}$ was unchanged by administration of this pharmacological blocker, it was clear that this change in P_{DA} was directly related to an atropine-induced increase in systemic vascular resistance (R_{sys} increasing from 0.32 to 0.35 kPa ml^{-1} kg min). Neither P_{DA} nor R_{sys} were affected by bretylium, atenolol or ICI 118551 (Fig. 2).

Net stress and chasing

Resting plasma levels of A and NA were both approx. 5 nmol l^{-1}, resulting in a A:NA ratio of approx. 1.1. The 90 s chase resulted in slightly higher post-stress levels of catecholamines than the 60 s net stress (A, 20.7±5.1 *vs* 15.0±7.0; NA, 14.0±5.0 *vs* 11.6±3.4), however, these differences were not significant (P=0.52 for A and P=0.70 for NA). This lack of a statistical difference was also true for A:NA ratios, which were 2.0 and 1.3 following the 90 s chase and 60 s net stressors, respectively (Table 2).

Catecholamine dose–response curves

In these experiments, we were aiming for maximum circulating catecholamine concentrations of approx. 25 nmol l^{-1} adrenaline and 14 nmol l^{-1} noradrenaline based on the injection of 0.4 μg kg^{-1} A and 0.2 μg kg^{-1} NA (dose 5). The plasma levels achieved with this dose at 2 min post-injection were 13.7 nmol l^{-1} for NA but only 11.5 nmol l^{-1} for A. Although this was an increase over resting levels of only 8.5 and 7 nmol l^{-1}, respectively, these values were

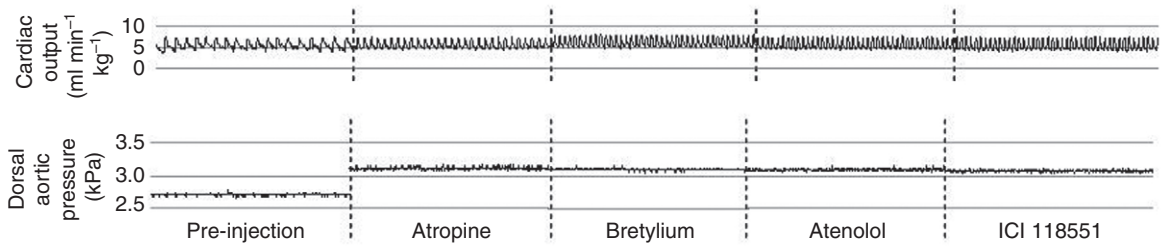

Fig. 1. Cardiac output (ml min^{-1} kg^{-1}) and dorsal aortic pressure (kPa) traces for one winter flounder. These traces are approximately 30 s in duration and were obtained 1 h after each drug injection. The drugs were sequentially injected every 1 h 30 min in the following order: atropine (1.2 mg kg^{-1}), bretylium (10 mg kg^{-1}), atenolol (213 μg kg^{-1}) and ICI 118 551 (250 μg kg^{-1}).

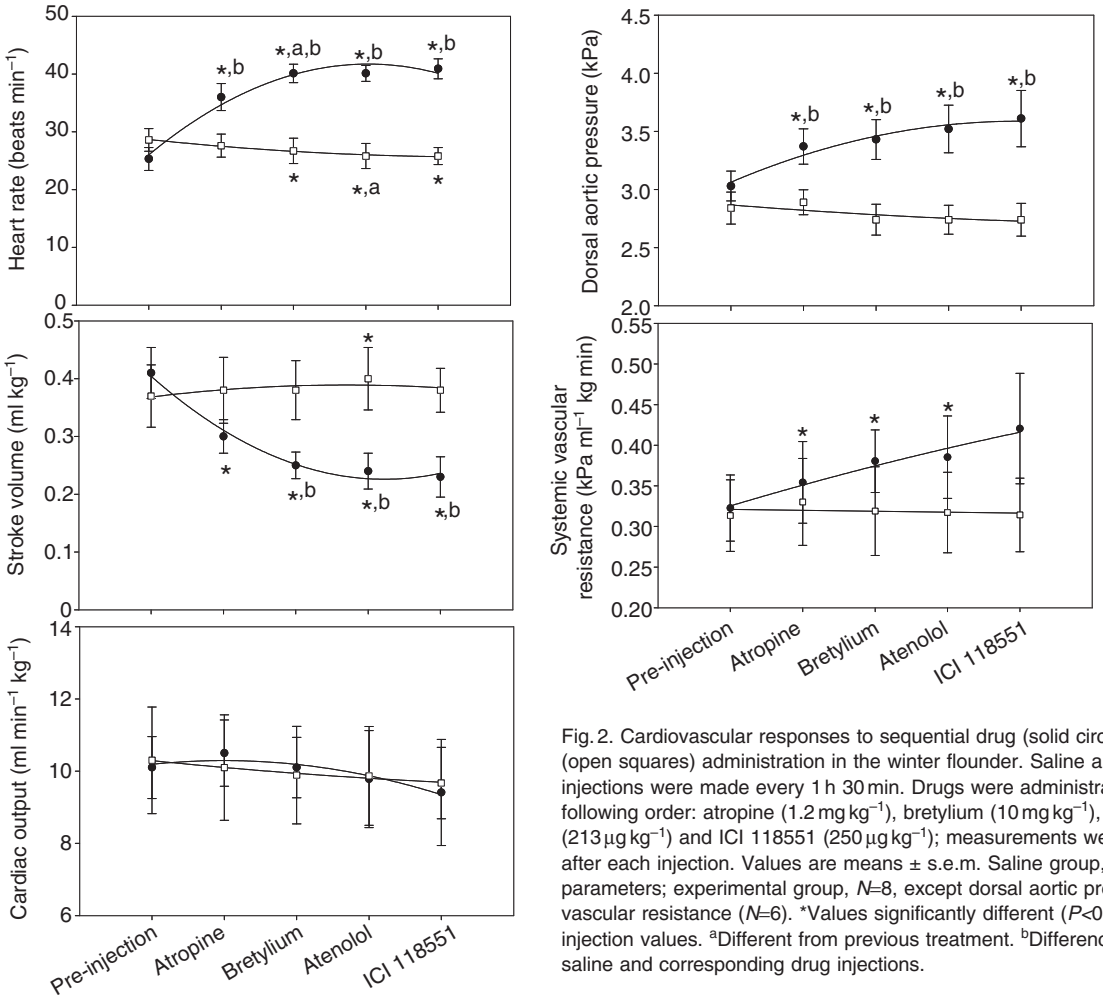

Fig. 2. Cardiovascular responses to sequential drug (solid circles) and saline (open squares) administration in the winter flounder. Saline and drug injections were made every 1 h 30 min. Drugs were administrated in the following order: atropine (1.2 mg kg^{-1}), bretylium (10 mg kg^{-1}), atenolol (213 μg kg^{-1}) and ICI 118551 (250 μg kg^{-1}); measurements were taken 1 h after each injection. Values are means ± s.e.m. Saline group, *N*=7 for all parameters; experimental group, *N*=8, except dorsal aortic pressure and total vascular resistance (*N*=6). *Values significantly different (*P*<0.05) from pre-injection values. [a]Different from previous treatment. [b]Difference between saline and corresponding drug injections.

significantly different from resting levels, and not significantly different from catecholamine concentrations measured after chasing and net stress (Table 2).

The injection of A and NA resulted in dose-dependent changes in cardiovascular parameters, with f_H decreasing, and $\dot{Q}$ and V_S increasing, by a maximum of 15, 30 and 27%, respectively (Fig. 3). However, because significant increases in $\dot{Q}$ (by 9%) and V_S (by 24%), and decreases in f_H (by 13%), were also associated with the injection of saline (Fig. 3), values were expressed as a percentage change from pre-injection, after subtracting the increase/decrease associated with saline injection. When this was done (Fig. 4), it was clear that the maximum post-injection increases in cardiovascular function were mainly an injection artefact. For instance, of the 27% increase in V_S reported after catecholamine injection, only 6% was actually due to the effects of A and NA on this cardiovascular parameter. In a similar fashion, only 10% of the increase in $\dot{Q}$ could be attributed to the direct effects of the injected catecholamines (Fig. 4). In contrast P_{DA} increased by 22% with the injection of A and NA, and this change was measured at the lowest catecholamine dose (0.1 μg kg^{-1} A and 0.05 μg kg^{-1} NA); increasing the dose by fourfold having only a marginal additional influence on P_{DA}.

Table 1. Resting cholinergic and adrenergic tone in different teleost species

Species	Temperature (°C)	Resting cholinergic tonus (%)	Resting adrenergic tonus (%)
Pleuronectes americanus*	**8**	**26.1**	**–11.9**
Pollachius pollachius[†]	11–12	19.7	33.2
Labrus bergylta[†]	11–12	33.9	15.4
Zoarces viviparus[†]	11–12	18.9	67.1
Myoxocephalus scorpius[†]	11–12	11.1	25.3
Sparus aurata[‡]	16	22.4	12.8
Gadus morhua[‡]	10	21.3	12.9
Gadus morhua[§]	10–12	37.7	21.1

*Present study (winter flounder); [†]Axelsson et al., 1987 (pollack, ballan wrasse, eel pout and short-spined scorpion); [‡]Altimiras et al., 1997 (Mediterranean sea bream and Atlantic cod); [§]Axelsson, 1988 (Atlantic cod).

Table 2. Plasma adrenaline and noradrenaline concentrations, adrenaline:noradrenaline ratio and total catecholamine concentrations, in the winter flounder at rest, after 60 s of net stress, after 90 s of chasing, and after an injection of catecholamines

	A (nmol l^{-1})	NA (nmol l^{-1})	A:NA	Total CA (nmol l^{-1})
Rest	4.6±0.7^{a}	5.2±1.0^{a}	1.1±0.3^{a}	9.8±1.1^{a}
Net stress	15.0±7.0^{b}	11.6±3.4^{b}	1.3±0.4^{a,b}	26.6±9.5^{b}
Chasing	20.7±5.1^{b}	14.0±5.0^{b}	2.0±0.4^{b}	34.6±8.4^{b}
Dose 5	11.5±2.3^{b}	13.7±3.6^{b}	1.0±0.1^{a}	25.2±5.8^{b}

A, adrenaline; NA, noradrenaline; A:NA, adrenaline:noradrenaline ratio; CA, total catecholamines (dose 5: 0.4 μg kg^{-1} of A and 0.2 μg kg^{-1} of NA). Values are presented as means ± s.e.m. Different superscript letters indicate a significant difference (P<0.05) between treatments.

In vitro experiments

Cardiac β-adrenoreceptors

The r^2 value for individual CGP-binding curves was always ≥0.86 (range=0.86–0.99; Fig. 5B), with non-specific binding ranging from 14.6 to 30.9% of total binding (Fig. 5A). When the binding data were converted into fmol mg^{-1} protein based on punch protein content (28.1±4.4 μg mg^{-1} tissue), the β-adrenoreceptor density (B_{max}) and ligand binding affinity (K_d) for the winter flounder myocardium were determined to be 252.8±45.6 fmol mg^{-1} protein and 1.02±0.11 nmol l^{-1}, respectively. Although atenolol (β_1-antagonist) was unable to displace [^{3}H]CGP from the flounder's ventricular β-adrenoreceptors, the β_2-antagonist ICI 118551 decreased [^{3}H]CGP binding beginning at approx. 10^{-7} mol l^{-1}; the IC_{50} value for ICI 118551 was 1.91×10^{-6} mol l^{-1} (Fig. 5C).

DISCUSSION

Resting cardiac function

Taking into consideration that cardiac function and P_{DA} are sensitive to acclimation temperature (Cech et al., 1976), resting cardiovascular variables in this study (at 8°C) correspond well with previous research on 10°C-acclimated winter flounder. For instance, Joaquim et al. (Joaquim et al., 2004), made the first direct measurements of cardiac function in flatfish, and reported resting V_S and $\dot{Q}$ values at 10°C of 0.47 ml kg^{-1} and 15.5 ml min^{-1} kg^{-1} (compared with 0.39 ml kg^{-1} and 10.4 ml min^{-1} kg^{-1} in the present study). Furthermore, several authors have reported f_H and P_{DA} values of approx. 35 beats min^{-1} and 3.5 kPa, respectively, at 10°C (Cech et al., 1976; Cech et al., 1977; Joaquim et al., 2004), values very similar to those measured in this study (27.5 beats min^{-1} and 2.9 kPa at 8°C,

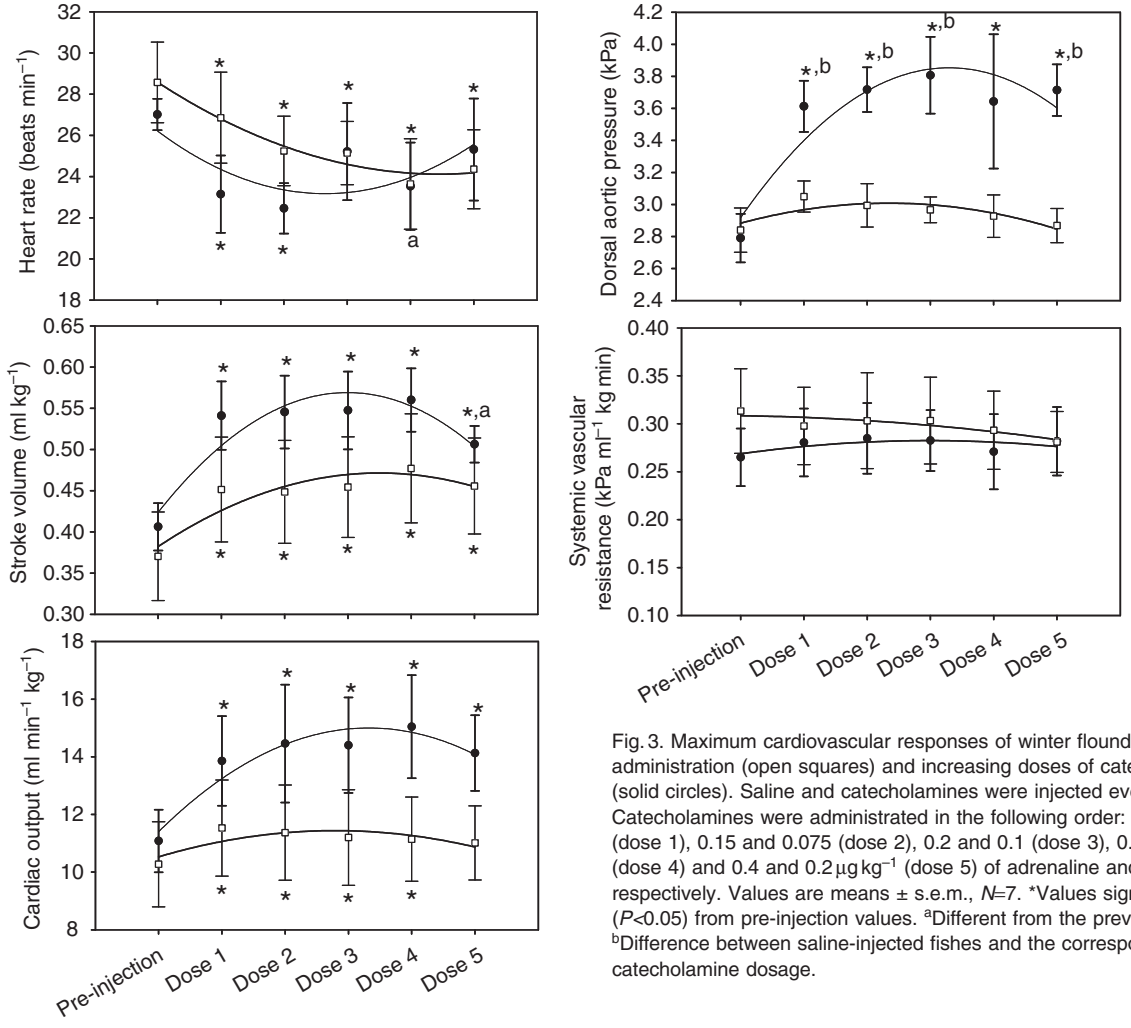

Fig. 3. Maximum cardiovascular responses of winter flounder to saline administration (open squares) and increasing doses of catecholamines (solid circles). Saline and catecholamines were injected every 1 h. Catecholamines were administrated in the following order: 0.1 and 0.05 (dose 1), 0.15 and 0.075 (dose 2), 0.2 and 0.1 (dose 3), 0.3 and 0.15 (dose 4) and 0.4 and 0.2 μg kg^{-1} (dose 5) of adrenaline and noradrenaline, respectively. Values are means ± s.e.m., N=7. *Values significantly different (P<0.05) from pre-injection values. [a]Different from the previous dosage. [b]Difference between saline-injected fishes and the corresponding catecholamine dosage.

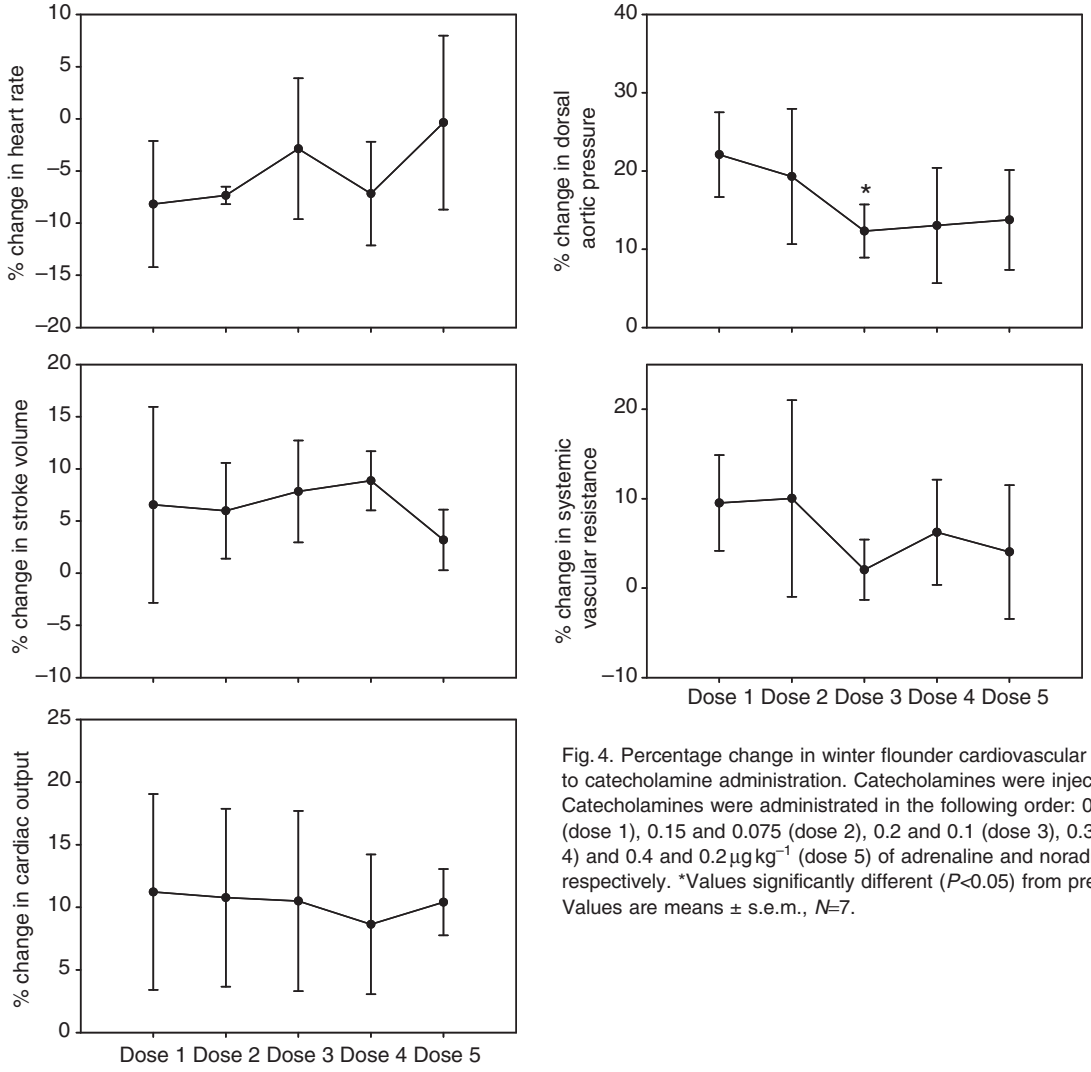

Fig. 4. Percentage change in winter flounder cardiovascular parameters due to catecholamine administration. Catecholamines were injected every 1 h. Catecholamines were administrated in the following order: 0.1 and 0.05 (dose 1), 0.15 and 0.075 (dose 2), 0.2 and 0.1 (dose 3), 0.3 and 0.15 (dose 4) and 0.4 and 0.2 μg kg^{-1} (dose 5) of adrenaline and noradrenaline, respectively. *Values significantly different (P<0.05) from pre-injection values. Values are means ± s.e.m., N=7.

respectively). By contrast, resting cardiovascular values for the flounder are generally lower than reported for more active species. This is not surprising, however, as significant inter-specific variation exists in cardiac function, and in general, active fishes have higher values for resting $\dot{Q}$ and V_S than benthic forms. For instance, in the rainbow trout, resting $\dot{Q}$ can range from 18 to 30.9 ml min^{-1} kg^{-1} and V_S from 0.29 to 0.6 ml kg^{-1} (at ~9–12°C) (Gamperl et al., 1994e; Thorarensen et al., 1996; Brodeur et al., 2001). The chinook salmon (*Oncorhynchus tshawytscha*) has resting values for $\dot{Q}$ of 35.8 ml min^{-1} kg^{-1} and for V_S of 0.63 ml kg^{-1} at 9–10°C (Gallaugher et al., 2001), and $\dot{Q}$ and V_S values of 27.5–35.5 ml min^{-1} kg^{-1} and 0.61 ml kg^{-1} have been reported for the Atlantic salmon (*Salmo salar*) at 9–12°C (Perry and McKendry, 2001; Dunmall and Schreer, 2003). However, the bottom dwelling lingcod [*Ophiodon enlongatus*; $\dot{Q}$ of 5.9–10.9 ml min^{-1} kg^{-1}; V_S of 0.13–0.37 ml kg^{-1} at 9–13°C: (Stevens et al., 1972; Farrell, 1981)] and the sea raven [*H. americanus*; $\dot{Q}$ of 18.8 ml min^{-1} kg^{-1} and V_S of 0.51 ml kg^{-1} at 6–8°C; (Axelsson et al., 1989)] have similar resting cardiovascular values as reported here for the flounder.

Neurohormonal control of cardiac function

Atropine induced a 42% increase in f_H, and based on this change in f_H we calculated that the cholinergic contribution to resting heart rate at 8°C was 26.1%. This degree of cholinergic tonus is equivalent to that reported for other teleosts at similar temperatures (see Table 1). For instance, the eel pout (*Zoarces viviparous*) has a resting cholinergic tonus of 18.9% (Axelsson et al., 1987) and cholinergic tonus on the cod heart is between 21.3 and 37.7% at 10–12°C (Altimiras et al., 1997; Axelsson, 1988).

Surprisingly, bretylium caused an additional positive chronotropic effect in the winter flounder that lasted for at least 1 h post-injection, and this resulted in a negative resting adrenergic tonus (–11.9%). This finding is in clear contrast to most previous studies on fish which have reported values for resting adrenergic tonus from ~13% for the sea bream (*Sparus aurata*) and cod (Altimiras et al., 1997) to 67.1% for the eel pout (Axelsson et al., 1987) (Table 1). Furthermore, the positive chronotropic effect induced by bretylium was not expected since earlier studies suggest that pleuronectids lack myocardial adrenergic innervation. For example, fluorescent histochemical studies in the greenback flounder (*Rhombosolea tapirina*) failed to find adrenergic cardiac nerves, and in isolated heart preparations from this species, stimulation of the vagus nerve had both negative inotropic and chronotropic effects (Donald and Campbell, 1982). In addition, in isolated plaice (*Pleuronectes platessa*) hearts, electrophysiological studies revealed that only cholinergic fibres run in the cardiac branch of the vagus, and that bretylium had no effect on f_H in vagally-stimulated isolated hearts (Cobb and Santer, 1973). However, there are several potential

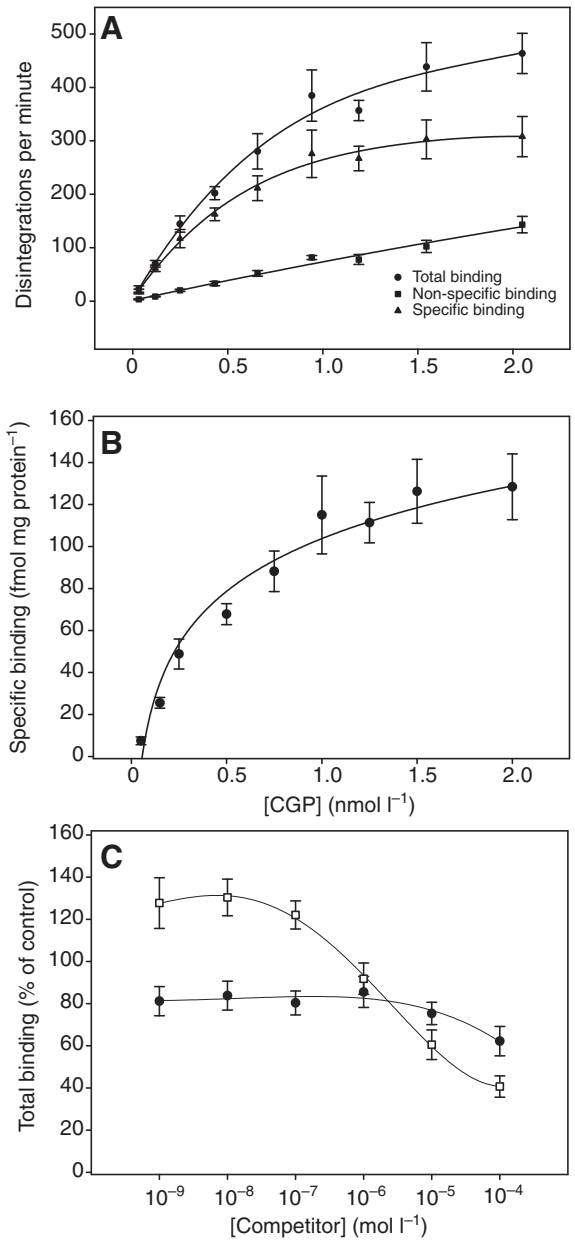

Fig. 5. (A) Total, non-specific and specific binding of [^{3}H]CGP-12177 to ventricular β-adrenoreceptors in the winter flounder; timolol (10^{-5} nmol l^{-1}) was used as the competitor (N=8). (B) Specific binding of [^{3}H]CGP-12177 to ventricular β-adrenoreceptors in winter flounder (y=36.2 Ln(x)+103.8; r^2=0.963; N=8). (C) Comparison of the ability of atenolol (β_1-antagonist; solid circles) and ICI 118551(β_2-antagonist, open squares) to displace [^{3}H]CGP-12177 from ventricular β-adrenoreceptors in the winter flounder (N=6). ICI 118551 IC_{50}=1.91×10^{-6} mol l^{-1}. Values represent means ± s.e.m.

explanations for the increase in f_H seen in the winter flounder following the injection of bretylium. First, unlike other pleuronectids, this species might have cardiac sympathetic innervation, and thus the positive chronotropic effect could be the result of an initial release of catecholamines from sympathetic nerve endings. This bretylium sympathomimetic effect has been demonstrated in both sand flathead (*Platycephalus bassensis*) isolated heart preparations (Donald and Campbell, 1982) and in Atlantic cod *in situ* heart preparations (Axelsson, 1988). Second, it is possible that the flounder has cardiac α-adrenoreceptors, and that basal adrenergic tone has a negative chronotropic influence in this species. In this scenario, bretylium would have increased heart rate by inhibiting catecholamine release from adrenergic nerve terminals. Such an inhibitory adrenergic effect, caused by the stimulation of α-adrenoreceptors, has been demonstrated in the hearts of the perch [*Perca fluviatilis*, 15 and 24°C (Tirri and Ripatti, 1982)] and eel [*Anguilla anguilla*, 8°C (Peyraud-Waitzenegger et al., 1980)]. Nonetheless, previous studies have not found any evidence of α-adrenoreceptors in atrial preparations from the rainbow trout and flounder (*P. flesus*) at 8°C (Ask, 1983), and thus, it is unlikely that α-adrenoreceptors were playing a significant role in the regulation of cardiac function in the winter flounder at 8°C. Third, as in mammals (Heissenbuttel and Bigger, 1979; Fallen, 1998), bretylium administration could have caused an initial catecholamine release from peripheral adrenergic nerve terminals into the circulation, elevating f_H. This explanation, however, also seems unlikely as catecholamines are rapidly cleared from the circulation of fishes (Gamperl et al., 1994b) and no significant increase in plasma catecholamines was found in the cod after bretylium injection (Axelsson and Nilsson, 1986). Furthermore, our dose–response curves show that the flounder heart is not very responsive to circulating catecholamines (e.g. see Figs 3 and 4), and that blocking the flounder's β-adrenoreceptors with atenolol and ICI 118551 did not mitigate the positive chronotropic effect associated with bretylium injection (Fig. 2). Unfortunately, at present, we cannot provide a definitive answer as to why bretylium induced a positive chronotropic effect in the flounder heart.

As mentioned above, the β_1 and β_2-adrenergic blockers atenolol and ICI 118551 had no effect on resting cardiovascular function in the winter flounder. This result indicates that neither circulating or endogenous catecholamines support resting cardiac function in this species of flatfish. This result is surprising given that other fish species that lack cardiac innervation (e.g. myxinoids), or whose vagus is composed solely of cholinergic nerves (most elasmobranchs), rely heavily on circulating and endogenous catecholamines for the modulation of cardiac function (Axelsson et al., 1990; Johnsson et al., 1996; Agnisola et al., 2003). However, it is consistent with the findings of other aspects of our investigation into the mechanisms controlling cardiac function in this species. Specifically, we show that the flounder heart is not very sensitive to increases in circulating catecholamines achieved by bolus injections of A and NA (Figs 3 and 4), and that β-adrenoreceptors in the flounder heart have a very low affinity (K_d for [^{3}H]CGP 12177 of 1.02 nmol l^{-1}).

Intrinsic heart rate in the flounder was calculated to be 40.9 beats min^{-1} at 8°C. This value is similar to the intrinsic f_H reported for the cod [36.6 beats min^{-1}, 10–12°C, (Axelsson, 1988)], and is in the middle of the range of values reported by Axelsson et al. (Axelsson et al., 1987) for seven different teleost species at 11–12°C; i.e. from approx. 30 beats min^{-1} for the tadpole fish (*Ranice raninus*) to as high as ~60 beats min^{-1} in the five-bearded rockling (*Ciliata mustela*).

Plasma catecholamine levels following net stress and chasing

The low resting levels of plasma catecholamines found in the winter flounder (A and NA concentrations of 4.6 and 5.1 nmol l^{-1}, respectively) are comparable to values reported for other teleosts, and indicate that our fish had recovered fully from surgery. For example, resting levels of A and NA in cod are reported to range from 2.5–4 and 4–5 nmol l^{-1}, respectively (Axelsson and Nilsson, 1986; Butler et al., 1989), and circulating catecholamine concentrations in resting rainbow trout are also <5 nmol l^{-1}

Table 3. Plasma adrenaline, noradrenaline, total catecholamines concentrations and haematocrit in different teleost species after chasing

Species	A (nmol l^{-1})	NA (nmol l^{-1})	Total CA (nmol l^{-1})	Hct (%)	Type of stress
Pleuronectes americanus*	**21**	**14**	**35**	**21**	**90 s chase**
Tautogolabrus adspersus†	80	23	103	35	90 s chase
Macrozoarces americanus†	58	41	99	28	90 s chase
Osmerus mordax mordax†	25	26	48	30	90 s chase
Mallotus villosus†	148	149	297	28	90 s chase
Gadus morhua†	188	47	235	28	90 s chase
Hemitripterus americanus‡	8	8	16	–	1 min air exposure followed by 1 min chase
Oncorhynchus mykiss§	179.7	51	230.7	–	10 min chase (freshwater)
Oncorhynchus mykiss§	88.1	19.5	107.6	–	10 min chase (seawater)
Oncorhynchus mykiss§	~30	~37	67	~40	6 min chase
Platichthys stellatus¶	~21	~30	51	~15	10 min chase

A, adrenaline; NA, noradrenaline; CA, total catecholamines; Hct, haematocrit.
*Present study (winter flounder); †Costa and Gamperl, unpublished data (cunner, ocean pout, Atlantic rainbow smelt, capelin, Atlantic cod); ‡Vijayan and Moon, 1994 (sea raven); §Tang and Boutilier, 1988 (rainbow trout held in freshwater and seawater); ¶Milligan and Wood, 1987 (rainbow trout and starry flounder).

[2.2–2.6 nmol l^{-1} for A and 2.7–3.3 nmol l^{-1} for NA (Milligan and Wood, 1987; Tang and Boutilier, 1988)].

When the flounder was exposed to net stress, plasma A and NA concentrations increased by 3.2-fold and 2.2-fold, respectively. These increases were very similar to those recorded after the flounder was chased to exhaustion (elevations in plasma A levels by 4.5-fold and in NA by 2.7-fold; Table 2), but are considerably lower than those recorded for many other teleosts. For example, after 90 s of chasing, plasma A and NA levels measured in the winter flounder were 20.7 and 14 nmol l^{-1}, respectively, whereas, using a similar chasing protocol, I. Costa and A.K.G. (unpublished data) found values as high as 188 (A) and 47 nmol l^{-1} (NA) in cod and 148 (A) and 149 nmol l^{-1} (NA) in capelin (Table 3). Our results for the winter flounder are consistent with those for the starry flounder following a 10 min chase to exhaustion [A to ~30 and NA to 37 nmol l^{-1} (Milligan and Wood, 1987)] and for the sea raven (*H. americanus*) after 1 min of air exposure followed by 1 min of chasing [total catecholamine levels ~16 nmol l^{-1} (Vijayan and Moon, 1994)], and add support to the notion that benthic/sluggish species have low post-stress circulating catecholamine levels. However, we must also caution that this is only a generalization and that exceptions do exist; I. Costa and A.K.G. (unpublished data) found total plasma catecholamine levels of 48 nmol l^{-1} for the active swimming Atlantic rainbow smelt (*Osmerus mordax mordax*), a value only approximately one-half of that measured in the benthic dwelling eel pout (*Macrozoarces americanus*; 58 nmol l^{-1} A and 41 nmol l^{-1} NA; Table 1).

Catecholamine dose–response curves and cardiac β-adrenoreceptors

The bolus administration of A and NA, at doses (up to 0.4 μg kg^{-1} A and 0.2 μg kg^{-1} NA) that resulted in circulating catecholamine concentrations typical of post-chase levels, did not affect flounder cardiovascular function (Fig. 4). This result agrees with recent experiments on cod where *in vivo* cardiac function was only marginally influenced at A doses below 4 μg kg^{-1} (L. H. Petersen and A.K.G., unpublished data), and where *in situ* hearts only showed marginal improvements in maximum pumping capacity and power output even when exposed to 200 nmol l^{-1} A (G. J. Lurman, L. H. Petersen, H. O. Portner and A.K.G., unpublished). However, it contrasts with the results of Gamperl et al. (Gamperl et al., 1994d) for the rainbow trout where the injection of A at doses as low as 0.2 μg kg^{-1} increased $\dot{Q}$ and V_S by approx. 33%. This diminished adrenergic sensitivity in flounder, as compared with rainbow trout, hearts is clearly not due to a reduced number of cardiac β-adrenoreceptors, as β-adrenoreceptor density in the flounder ventricle (B_{max}, 252.8 fmol mg^{-1} protein) is the highest ever reported for a teleost species, and six- to 12-fold higher than reported for the rainbow trout (23–40 fmol mg^{-1} protein) (Gamperl et al., 1994a; Olsson et al., 2000). A more likely explanation is that the injected concentrations were not sufficient to stimulate the flounder heart. This conclusion is based on two pieces of evidence: (1) flounder (*P. flesus*) ventricular strips at 10°C respond to 1 μmol l^{-1} of A by increasing contractile force by approx. 120% (Lennard and Huddart, 1992); and (2) although the density (B_{max}) of ventricular β-adrenoreceptors is very high compared with other teleosts, their binding affinity for [^{3}H]CGP is 1.02 nmol l^{-1}. This latter value is approx. 3–10 times greater than that measured for a variety of other fish species [0.13–0.36 nmol l^{-1} (Olsson et al., 2000)].

Although unusual, a very low receptor affinity for stress hormones is not unique amongst fishes. For example, gill cortisol receptors in the chub (*Leuciscus cephalus*) have a K_d eightfold higher than those in rainbow trout, probably to compensate for the extremely high levels of cortisol found in the blood of this species [at rest, five- to 10-fold higher than trout (Pottinger et al., 2000)]. Why the β-adrenoreceptors on the flounder ventricle have such a high K_d is not obvious from the research conducted here (but see below), especially given that resting plasma concentrations of A and NA are low, and typical of those measured in other teleosts. However, it is possible that the high K_d value of flounder heart β-adrenoreceptors is related to this species' life history. The flounder has a benthic and inactive lifestyle (Pereira et al., 1999), a limited aerobic capacity (Duthie, 1982; Lefrançois and Claireaux, 2003; Priede and Holliday, 1980), generally swims intermittently (He, 2003), and appears to find even slow swimming very demanding (Joaquim et al., 2004). Thus, it is reasonable to assume that the flounder's adrenergic and cardiovascular systems are not designed to respond to chasing/exercise, an unlikely situation for the flounder in its natural environment. Interestingly, flounder typically bury themselves several cm deep (i.e. up to 10–15 cm) in soft sediments (sand and mud), and under these conditions gill ventilation may be difficult (Cech et al., 1977; Nonnotte and Kirsch, 1978) and very low oxygen concentrations may be encountered (Fletcher, 1975; Duthie, 1982; Pereira et al., 1999). In experiments using *in situ* heart preparations, Mendonça et al. (Mendonça et al., 2007) reported that the winter flounder heart has a maximum V_S (2.3 ml g^{-1} ventricle) significantly greater than the cod (1.3) and the Atlantic salmon (1.4), and suggested that this elevated maximum V_S might be important under conditions of severe hypoxia. This hypothesis is supported by recent *in vivo* data (P.C.M. and A.K.G., unpublished data)

showing that flounder acclimated to 8°C increased V_S by 40% when the water O_2 saturation was lowered from 30 to 20%, and thus it is probable that severe hypoxia is the type of stress/challenge that would promote a release of catecholamines from the chromaffin cells large enough to stimulate flounder myocardial β-adrenoreceptors. In fact, this idea is supported by recent research on Atlantic cod showing that circulating catecholamine levels during severe hypoxia [A ~350 nmol l^{-1} and NA ~60 nmol l^{-1} at 13% water O_2 saturation (L. H. Petersen and A.K.G., unpublished)] are approximately twice those following exhaustive exercise (A of 188 nmol l^{-1}, NA of 47 nmol l^{-1}; I. Costa and A.K.G., unpublished data), and that maximal adrenergic stimulation offsets the adverse effects of hypoxia, and the concomitant effects of hyperkalemia and acidosis, on performance of *in situ* trout heart at 10°C (Hanson et al., 2006).

Based on the inability of the β_1-antagonist atenolol to displace [^{3}H]CGP (β-adrenergic antagonist) from flounder's ventricular β-adrenoreceptors, and the IC_{50} value for the β_2-antagonist ICI 118551 (1.91×10^{-6} mol l^{-1}), it appears that winter flounder ventricular β-adrenoreceptors are predominantly of the β_2 subtype. This conclusion contrasts with the previously held belief that the flounder heart has equal affinity for A and NA (Ask, 1983), but agrees with Cobb and Santer (Cobb and Santer, 1973) who showed that the isolated heart of the flounder *P. platessa* is more sensitive to A than to NA, and with Gamperl et al. (Gamperl et al., 1994a) who characterized the ventricular β-adrenoreceptors of the rainbow trout heart as β_2. Nonetheless, the high K_d measured for these receptors (1.02 nmol l^{-1} CGP) is difficult to reconcile with previous studies on fish which report K_d values in the range of ~0.1–0.4 nmol l^{-1} (Gamperl et al., 1994a; Olsson et al., 2000). One possible explanation for the high K_d value measured for β-adrenoreceptors of the flounder heart is that they are functionally uncoupled from adenylate cyclase (Hausdorff et al., 1990). However, we feel that this is unlikely as resting levels of circulating catecholamines in the flounder are typical of those measured in other teleosts, the repeated injection of catecholamines into rainbow trout does not alter cardiac β-adrenoreceptor affinity (Gamperl et al., 1994a) and exposure to β-adrenoreceptor agonists for more than a month only resulted in an increase in the K_d of rainbow trout muscle β-adrenoreceptors of 2-fold [from ~0.2 to 0.5 nmol l^{-1} (Lortie et al., 2004)]. A more probable explanation for the high K_d value reported here for flounder cardiac β-adrenoreceptors is that the flounder heart has a significant population of β_3-adrenoreceptors. These receptors have been found in the hearts of both the rainbow trout (Nickerson et al., 2003) and European eel (Imbrogno et al., 2006), and are activated at higher catecholamines concentrations than β_1/β_2-adrenoreceptors (Gauthier et al., 2007). Furthermore, in trout erythrocytes, β_3-adrenoreceptor binding properties resemble β_2-adrenoreceptors more than β_1 (Nickerson et al., 2003), and consequently the displacement of [^{3}H]CGP by ICI 118551 in the flounder heart (see Fig. 5C) may be related to the presence of β_2 and/or β_3 subtypes. β_3-receptor stimulation induces negative inotropic effects in most mammals (e.g. see Gauthier et al., 1999; Gauthier et al., 2007) and in the eel (Imbrogno et al., 2006), and it has been recently shown by Imbrogno et al. (Imbrogno et al., 2006) and Angelone et al. (Angelone et al., 2008) that stimulation of β_3-adrenoreceptors mediates negative inotropy and lusitropy (i.e. their stimulation allows the heart to relax slower) through the induction of endothelial nitric oxide synthase-derived nitric oxide signalling. Whether these receptors also exist in the flounder heart, and what role they might play in mediating cardiac function in this species, can only be answered through further research. However, it has been suggested that these receptors play a 'protective role' by preventing excessive β_1/β_2 stimulation of the mammalian myocardium (Gauthier et al., 2007; Angelone et al., 2008).

Perspectives

This study provides valuable new insights into the nervous and humoral control of cardiac function in the winter flounder. For example, our results indicate that neural and humoral adrenergic mechanisms do not support resting cardiac performance in 8°C-acclimated flounder, and that increases in plasma catecholamines associated with net stress and chasing are not sufficient to stimulate cardiac β-adrenoreceptors. They also show that although the flounder ventricle is populated with an unusually large number of β-adrenoreceptors, these receptors have a very low binding affinity. These results are not easily explained on the basis of what is known about cardiovascular control/physiology in other fishes, but appear to fit well with the winter flounder's life history and other data on aspects of this species' cardiac function (e.g. Joaquim et al., 2004; Mendonça et al., 2007). Furthermore, they lead to several testable hypotheses. Specifically, we propose that this species' muted cardiac response to catecholamine stimulation is related to the existence of a significant population of myocardial β_3-adrenoreceptors, that circulating catecholamines only reach concentrations capable of stimulating these receptors under conditions such as severe hypoxia (e.g. when flounder are buried in soft sediments), and that β_3-adrenoreceptor stimulation promotes negative inotropism and lusitropism that balances the positive stimulation provided by β_2-receptors.

LIST OF ABBREVIATIONS

A	adrenaline
B_{max}	cell surface β-adrenoreceptor density
f_H	heart rate
IC_{50}	concentration of ligand that reduced [^{3}H]CGP binding by 50%
K_d	dissociation constant (for [^{3}H]CGP 12177)
NA	noradrenaline
P_{DA}	dorsal aortic pressure
$\dot{Q}$	cardiac output
R_{sys}	systemic vascular resistance
U_{crit}	critical swimming speed
V_S	stroke volume

This work was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant to A. Kurt Gamperl. Paula C. Mendonça was supported by a doctoral fellowship SFRH/BD/8082/2002 from the Portuguese Foundation for Science and Technology (FCT) co-financed through POCI 2010 (Programa Operacional Ciência e Inovação) and FSE (Fundo Social Europeu). We are grateful to Dr Trevor Avery for statistical advice, to Isabel Costa for assistance with plasma catecholamine measurements, and to Linda Hanson for help with analysis of the β-adrenoreceptor data.

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