

## Intrinsic autoregulation of cardiac output in rainbow trout (*Oncorhynchus mykiss*) at different heart rates

Jordi Altimiras\* and Michael Axelsson

Department of Zoology, University of Göteborg, Box 463, S-405 30 Göteborg, Sweden

\*Author for correspondence at present address: Department of Biology, IFM, Linköpings Universitet, SE-58183 Linköping, Sweden  
(e-mail: jordi@ifm.liu.se)

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### Summary

Intrinsic regulation of the heart in teleosts is partly driven by central venous pressure, which exerts a modulatory role on stroke volume according to the well-known Frank–Starling mechanism. Although this mechanism is well understood from heart perfusion studies, less is known about how this mechanism operates *in vivo*, where heart rate varies markedly. We used zatebradine, a bradycardic agent, to attain resting heart rates in surgically instrumented animals. A dose of zatebradine of  $2.79 \pm 0.47$  mg l<sup>-1</sup> decreased heart rate by half, from  $44.4 \pm 4.19$  beats min<sup>-1</sup> to  $22.1 \pm 1.9$  beats min<sup>-1</sup>. Zatebradine had no significant effect on the peripheral vasculature and no inotropic effects, so was a suitable pharmacological agent with which to manipulate heart rate. When heart rate halved, cardiac output dropped to  $87.5 \pm 4.6\%$  of the control value, due to the concomitant increase in stroke volume to  $165 \pm 13\%$ . *In vivo* recordings

of venous pressure at varying heart rates indicated that the partial compensation in cardiac output was possible through an increase in pressure in the sinus venosus, from  $-0.06 \pm 0.04$  kPa at a control heart rate of  $58.3 \pm 3.5$  beats min<sup>-1</sup> ( $N=10$ ) to  $0.07 \pm 0.05$  kPa after injection of zatebradine ( $4$  mg kg<sup>-1</sup>). The operation of the so-called time-dependent autoregulatory mechanism was further demonstrated in perfused hearts. The positive pressures recorded in the sinus venosus at low heart rates coincident with non-invasive measurements in trout suggest that atrial filling in trout is more dependent on the build-up of pressure in the venous circulation (*vis-à-tergo* filling) than a suction mechanism during ventricular contraction (*vis-à-fronte* filling).

Key words: venous pressure, heart rate, Frank–Starling mechanism, zatebradine, rainbow trout, fish, *Oncorhynchus mykiss*.

### Introduction

Intrinsic regulation of the teleost heart is greatly dependent on central venous pressure, which exerts a modulatory role on stroke volume according to the well-known Frank–Starling mechanism. This mechanism is well characterized *in situ* (Farrell et al., 1982; Farrell, 1991) and results from perfused heart studies have been extrapolated to cardiac function in the intact animal.

Heart perfusion studies, however, operate at rates well above the *in vivo* range of resting heart rate (Altimiras and Larsen, 2000) because the inhibitory cholinergic influence on the heart is abolished. At the lower *in vivo* heart rates, the loading conditions of the heart will differ due to a longer filling time (Farrell and Jones, 1992), which in turn is expected to increase venous pressure.

This prediction has not been demonstrated experimentally due to technical limitations in recording pressure in the sinus venosus (*Ps<sub>v</sub>*) in teleosts. Such a study, however, could shed light on two separate aspects of the cardiac physiology of teleosts. First, it could explain the shift from *vis-à-fronte* atrial filling to *vis-à-tergo* filling (Farrell, 1991). The unique *vis-à-*

*fronte* filling mechanism observed in some fish species relies on the generation of negative intrapericardiac pressures associated with the elastic recoil of the ventricle during diastole. This mechanism differs from *vis-à-tergo* filling, which depends on the build-up of pressure in the central veins. Second, it could explain the role of venous pressure in modulating cardiac output through the interdependence between heart rate and stroke volume.

The aim of the study was to validate a new technique for measuring *Ps<sub>v</sub>*, and to study *Ps<sub>v</sub>* in trout at varying heart rates. Since truly resting heart rates were unattainable, a novel pharmacological approach to manipulate heart rate using the bradycardic agent zatebradine was developed and validated.

### Materials and methods

#### Animal handling

Rainbow trout *Oncorhynchus mykiss* Walbaum were obtained from a local fish farm. Fish were kept in 2 m<sup>3</sup> tanks

at 15°C in the semi-open freshwater circulation system of the department and fed twice a week.

### Series I. Effects of zatebradine on cardiovascular parameters in vivo

Eight fish (580±53 g body mass) were used. The fish were anaesthetized in a solution of MS-222 (100 mg l<sup>-1</sup>; Sigma) buffered with sodium bicarbonate (200 mg l<sup>-1</sup>) until breathing movements ceased, and placed ventral side up on an operating sling. The gills were continuously irrigated with aerated water containing a diluted solution of MS-222 (75 mg l<sup>-1</sup>). A polyethylene cannula (PE50, Clay Adams; Becton Dickinson, Sparks, MD, USA) filled with heparinized (100 i.u. ml<sup>-1</sup>) 0.9% NaCl was implanted into the dorsal aorta to measure blood pressure (*P*<sub>DA</sub>) as previously described (Hughes et al., 1983). The cannula was secured with a suture on the back of the animal. In order to measure cardiac output  $\dot{Q}$  (=ventral aortic blood flow), the ventral aorta was exposed through an incision on the left side of the isthmus. A cuff-type pulsed Doppler flow probe (Iowa Doppler Products, Iowa City, IA, USA) with an internal diameter of 2.2–3.0 mm was placed around the ventral aorta and the lead from the probe was secured with two skin sutures.

After surgery, the animals were transferred to a holding chamber and allowed to recover for 24–36 h. The flow probes were connected to a Directional Pulsed Doppler Flowmeter (Model 545-4C, Iowa University, USA). The dorsal aortic cannula was connected to a Statham P23 (Hato Rey, Puerto Rico) pressure transducer connected to a bridge amplifier channel of a recorder (Grass Instruments, Quincy, MA, USA). The pressure transducer was calibrated against a static water column. Heart rate (*f*<sub>H</sub>) was obtained from the phasic blood flow signal using a Grass tachograph (model 7P44D).

The flow, pressure and tachograph signals were fed into a computer and stored to disk at 10 Hz using a custom-made program (Labview v.5.1, National Instruments, Austin, TX, USA).

The experimental protocol consisted of a control period followed by serial injections of zatebradine hydrochloride (Boehringer Ingelheim, Skärholmen, Sweden), corresponding to cumulative doses of zatebradine of 0.5, 1, 2, 4, 6 and 8 mg kg<sup>-1</sup>. A 30 min recording period was taken post-injection. The main decrease in heart rate occurred within the first 10 min post-injection.

### Series II. Twitch force and rate of force development in ventricular strips

Six fish were used (450–550 g in body mass). Fish were killed by a sharp blow to the head and the heart was rapidly excised from the animal and placed on a chilled Petri dish, where three longitudinal strips under 1 mm width were obtained from the ventral ridge of the pyramidal heart.

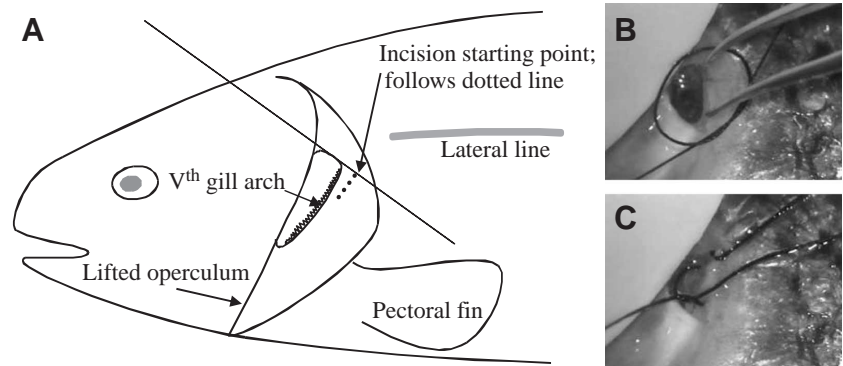
The strips were mounted for measurements of twitch force and rate of contraction using force transducers (Grass, FT-103) connected to a 4-channel bridge amplifier unit (Somedic, SenseLab 4CHAMP, Hörby, Sweden). The signals were stored digitally at 20 Hz for further analysis using a custom-made program (LabView 5.1, National Instruments). The Ringer solution was identical to the one used in the heart perfusion experiments and was also bubbled with 80 kPa O<sub>2</sub>/balance N<sub>2</sub>.

The strips were stretched to 90% of maximal contraction, paced at 0.2 Hz throughout the experiment and allowed to stabilize for 1 h. Following a 20 min control recording, the Ringer solution was quickly changed and the first concentration of zatebradine was tested. The three strips were run simultaneously, one being used as control and the other two as treatments. The concentrations of zatebradine tested were 0.5, 1, 2, 5 and 10 mg l<sup>-1</sup>.

### Series III. Effects of zatebradine on venous pressure

Ten fish, mass 300–500 g, were used in the experiment. The animals were instrumented with a dorsal aortic catheter for measurement of heart rate and drug injection as described for Series I. Following this procedure, a catheter was non-occlusively inserted in the left ductus of Cuvier (LDC) and forwarded to the sinus venosus. The surgical procedure was as follows. The operculum and the gills were retracted to expose the gill-free V<sup>th</sup> branchial arch and a 1 cm incision was made parallel to this arch (see Fig. 1 for a graphical view). The incision was initiated on top of the cleithrum bone and was followed towards the ventral edge of the bone. The LDC was exposed at this location. It was noticed that the motor nerve to the left pectoral fin runs on top of the LDC and this was used as an anatomical landmark. A loose pocket of the LDC wall was gently pulled and held with 3-0 suture thread (Fig. 1C). A loose thread was placed downstream and a small cut was made between the threads. A PE-50 catheter (with a bubble 35 mm

Fig. 1. Anatomical details of the surgical implantation of a cannula in the sinus venosus. (A) Schematic drawing showing the position of insertion in the V<sup>th</sup> gill arch. (B) Picture showing the thin-walled left duct of Cuvier (LDC) bulging from the incision site. (C) Picture showing the two silk snares in place before the cannulation. The tied snare (top one) is used to pull the vessel wall during the procedure, the untied snare (bottom one) is used to secure the cannula to the LDC after insertion.



from the tip) was inserted in the hole and advanced to the sinus venosus (15–20 mm) before secured tightly in place. The cannula was secured twice on the skin, once adjacent to the bubble on top of the cleithrum bone and once close to the dorsal fin. The position of the catheter tip was verified *post-mortem*.

This new procedure is similar to those previously used to measure the pressure in the ducts of Cuvier (Olson et al., 1997; Minerick et al., 2003), but allows the measurement of pressures in the sinus venosus.

Fish were allowed to recover for 24 h before the effects of three doses of zatebradine (1, 2 and 4 mg kg<sup>-1</sup>) on heart rate and venous pressure were recorded.

#### *Series IV. Heart rate-stroke volume relationship in the perfused heart*

Nine fish were used (505±24 g body mass, 469±31 mg wet ventricular mass). Fish were killed by a sharp blow to the head and transferred to an operating sling. Heparin (1 ml kg<sup>-1</sup>) was injected *via* the caudal vessels to prevent clotting of the blood during surgery. The heart was perfused *in situ* following previous protocols with a few variations (Farrell et al., 1986). Briefly, the abdominal cavity was opened, a double-bore cannulae (as shown in Franklin and Axelsson, 1994) was inserted in the sinus venosus through a hepatic vein and the perfusion of Ringer started from a reservoir. Other hepatic veins, if present, were ligated to prevent leakage. The gill arches were cut and Ringer flowed freely through the heart with each heart beat. The isthmus was cut, exposing the ventral aorta and the afferent branchial arteries, and a double bore cannula was inserted through the ventral aorta into the bulbus arteriosus. The entire fish was then transferred to a constant temperature stainless steel trough (15°C) filled with 0.9% NaCl. The cannula to the atrium was connected to two water-jacketed reservoirs with Ringer solution *via* a constant pressure device. The ventral aortic cannula was connected to an output pressure head. The composition of the perfusion solution was (in mmol l<sup>-1</sup>): 104.1 NaCl; 3.1 KCl; 0.9 MgSO<sub>4</sub>; 2.5 CaCl<sub>2</sub>. The buffer system was a Hepes–Tris mixture (5 mmol l<sup>-1</sup> Hepes adjusted to pH 7.8 with Tris to a final concentration in the perfusate of 5 mmol l<sup>-1</sup>). The following metabolic substrates were added: glucose, glutamate, fumarate and pyruvate (5 mmol l<sup>-1</sup> each). The perfusate was gassed with 80 kPa O<sub>2</sub>/balance N<sub>2</sub> using a Gas Mixing Flowmeter (model GF-3MP, Cameron Instruments, Port Aransas, TX, USA).

A tonic adrenergic stimulation (5 nmol l<sup>-1</sup> adrenaline bitartrate; Sigma) was maintained by pumping a 2.5 µmol l<sup>-1</sup> adrenaline-Ringer solution through a side port of the inflow tubing to the heart using a peristaltic pump (Minipuls 3, Gilson, Villiers le Bel, France). It has been established that tonic adrenergic stimulation is essential for the long-term viability of perfused hearts (Graham and Farrell, 1989). The flow rate of the pump was automatically adjusted to follow changes in cardiac output so that the dilution rate of the adrenaline solution was constant (1:500). The same system was used to

deliver a constant zatebradine concentration (1 mg l<sup>-1</sup>) to the heart.

Cardiac output was measured using an in-line flow probe (Transonic 2N, Ithaca, NY, USA; 2 mm internal diameter) coupled to a transit-time flowmeter (Transonic, T106). Preload and afterload pressures were measured using disposable pressure transducers (DPT6100, Peter von Berg Medizintechnik, Kirchseeon/Eglharting, Germany) connected to a 4-channel bridge amplifier unit (SenseLab 4CHAMP). Pressures were calibrated against static water columns and the calibration checked every 30 min. The signals were recorded on a Grass recorder (model 7WU), which also logged instantaneous heart rate using a tacograph unit (Grass, 7P44). In parallel, the data was digitally stored using a custom made program (LabView 5.1, National Instruments). Preload and afterload pressures, cardiac output and heart rate were averaged for 5 s and stored.

The experimental protocol consisted of a 30 min stabilization period, followed by a recording period, during which zatebradine-free perfusate (Control group, *N*=4) or perfusate with 1 mg l<sup>-1</sup> zatebradine (ZAT group, *N*=5) was supplied to the heart. The zatebradine dose was chosen after preliminary experiments. Larger doses (2, 3 and 4 mg l<sup>-1</sup>) elicited faster changes in *f*<sub>H</sub> but were unsuitable for prolonged exposure because the heart became arrhythmic before reaching a heart rate of 30 beats min<sup>-1</sup>, which was the lowest target *f*<sub>H</sub> intended in the experiment.

During the stabilization period, afterload was set to 5 kPa and cardiac output was adjusted to 30 ml min<sup>-1</sup> kg<sup>-1</sup> *via* changes in preload pressure. In the ZAT group, preload pressure was allowed to change as heart rate decreased following administration of zatebradine. The perfused hearts in the Control group were also subjected to the zatebradine after the 2 h control trial.

#### *Calculations and statistics*

In Series I, stroke volume (*V*<sub>S</sub>) was calculated as the ratio between  $\dot{Q}$  and *f*<sub>H</sub>. Systemic vascular resistance (*R*<sub>sys</sub>) was calculated as *P*<sub>DA</sub> divided by  $\dot{Q}$ , assuming that venous pressure was zero and did not change during the experimental protocol. It was also assumed that no significant changes in blood viscosity took place during the experiment.  $\dot{Q}$ , *V*<sub>H</sub> and *R*<sub>sys</sub> are presented as percent changes from the control value.

The dose–effect relationship of zatebradine (ZAT) on *f*<sub>H</sub> resembles that of a Michaelis–Menten enzymatic reaction if the effects are plotted as the decrease in *f*<sub>H</sub> from control values. Thus, a Levenberg–Marquardt non-linear fitting procedure was used to adjust the *f*<sub>H</sub> *versus* [ZAT] data for each animal to the Michaelis–Menten equation to obtain the concentration of zatebradine that induces half maximal effects on *f*<sub>H</sub>, referred here as [ZAT]<sub>0.5</sub>.

Control values of the different cardiovascular parameters were subsequently compared against those values at [ZAT]<sub>0.5</sub> for normalization purposes. If the [ZAT]<sub>0.5</sub> deviated more than 10% of the closest nominal [ZAT] used in the study, the values

for  $[ZAT]_{0.5}$  were linearly interpolated between the two values closest to  $[ZAT]_{0.5}$ .  $[ZAT]_{0.5}$  was in the range 1.5–3 mg l<sup>-1</sup>. Since the window around  $K_m$  in the Michaelis–Menten equation is highly linear, the error induced by interpolation is minimized.

In Series II, twitch force and maximal rate of contraction were determined as previously described (Hove-Madsen and Gesser, 1989).

In Series IV, power was calculated as:

$$\text{Power} = \frac{(P_{\text{out}} - P_{\text{in}}) \times \dot{Q}}{M_v}, \quad (1)$$

where Power is obtained in mW g<sup>-1</sup> if  $P_{\text{out}}$  (afterload pressure) and  $P_{\text{in}}$  (preload pressure) are in kPa,  $\dot{Q}$  is cardiac output in ml s<sup>-1</sup> and  $M_v$  is ventricular mass in g.

All data are presented as means  $\pm$  S.E.M.

Wilcoxon's signed-ranks test for paired samples (two-tailed) were used to evaluate the statistical significance of control and  $[ZAT]_{0.5}$  cardiovascular variables in Series I and III and the cardiac variables at different heart rates in Series IV. A Kruskal–Wallis non-parametric paired test was used to compare the effects of zatebradine in Series II. Asterisks in the figures indicate significant changes between paired samples. In the case of repeated tests, a modified Bonferroni procedure was used to reduce the risk of discarding a true null hypothesis (Holm, 1979).

## Results

### Series I. Cardiovascular parameters in vivo

Zatebradine is an effective bradycardic agent *in vivo*. From a control value of  $f_H = 44.4 \pm 2.3$  beats min<sup>-1</sup> ( $N=14$ ), a dose of 2 mg kg<sup>-1</sup> resulted in a 41% decrease in  $f_H$ . The effects plateaued at  $10.4 \pm 2.7$  beats min<sup>-1</sup> at the highest dose used (10 mg kg<sup>-1</sup>). This  $f_H$  is outside the physiological range of  $f_H$  for trout at 15°C (Fig. 2).  $[ZAT]_{0.5}$  was  $2.79 \pm 0.47$  mg l<sup>-1</sup>.

The effects of zatebradine were highly significant. At  $[ZAT]_{0.5}$ ,  $f_H$  decreased by 50%,  $P_{DA}$  decreased by 14% and  $\dot{Q}$  by 15%, while  $V_s$  increased by 66% and  $R_{sys}$  remained unchanged (Figs 2 and 3).

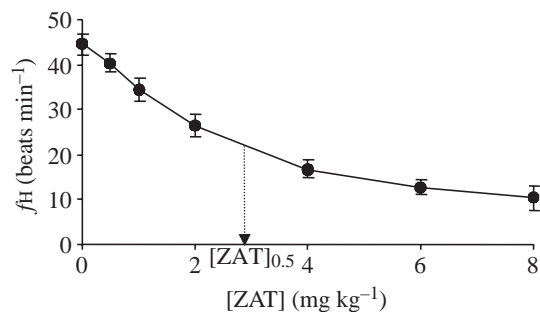


Fig. 2. Dose–response curve of the effect of zatebradine (ZAT) on heart rate  $f_H$ . An arrow indicates  $[ZAT]_{0.5}$ , the concentration of zatebradine at which heart rate was half the control value. Values are means  $\pm$  S.E.M. ( $N=14$ ).

Table 1. Effect of different doses of zatebradine on relative twitch force and rate of pressure development ( $dF/dt$ ) in trout ventricular strips

Dose (mg ml <sup>-1</sup> )	Twitch (%)	$dF/dt$ (%)
0 (Control)	100	100
0.5	102.6 $\pm$ 2.7	101.7 $\pm$ 3.2
1	105.3 $\pm$ 4.5	101.6 $\pm$ 5.4
2	105.1 $\pm$ 5.7	99.8 $\pm$ 6.3
5	104.8 $\pm$ 5.6	95.6 $\pm$ 6.1
10	99.1 $\pm$ 6.0	88.2 $\pm$ 5.9

Values are means  $\pm$  S.E.M. ( $N=6$ ).

### Series II. Twitch force and rate of force development in ventricular strips

The application of zatebradine to ventricular strips did not show any significant changes on twitch force or on the maximum rate of force development at any of the doses employed (Table 1).

### Series III. Venous pressure

The stepwise decrease in heart rate induced with zatebradine was coupled to a progressive increase in venous pressure. At a control  $f_H$  of  $58.3 \pm 3.5$  beats min<sup>-1</sup> ( $N=10$ ), venous pressure was subambient ( $-0.06 \pm 0.04$  kPa) and this increased significantly to  $-0.02 \pm 0.04$  kPa and  $0.07 \pm 0.05$  kPa after injection of zatebradine (2 mg kg<sup>-1</sup> and 4 mg kg<sup>-1</sup>, respectively) (Fig. 4).

### Series IV. Heart rate–stroke volume relationship in the perfused heart

The control experiments revealed little deterioration of  $f_H$  and  $P_{in}$  in the perfused heart after 2 h (Fig. 5).  $P_{in}$  increased from  $-0.023 \pm 0.013$  kPa ( $N=4$ ) to  $0.003 \pm 0.020$  kPa and  $f_H$  decreased from  $69 \pm 4$  beats min<sup>-1</sup> to  $59 \pm 3$  beats min<sup>-1</sup>.  $\dot{Q}$  decreased from  $28.1 \pm 1.2$  ml min<sup>-1</sup> kg<sup>-1</sup> to  $25.6 \pm 1.3$  ml min<sup>-1</sup> kg<sup>-1</sup>,  $V_s$  increased from  $0.41 \pm 0.03$  ml kg<sup>-1</sup> to  $0.44 \pm 0.03$  ml kg<sup>-1</sup> and power output decreased from  $2.52 \pm 0.18$  mW g<sup>-1</sup> to  $2.27 \pm 0.17$  mW g<sup>-1</sup> (not shown). In comparison, zatebradine-treated preparations (1 mg kg<sup>-1</sup>,  $N=7$ ) showed a significant change in preload and heart rate after 90 min perfusion. The decrease in  $f_H$  was almost linear down to 40 beats min<sup>-1</sup> and slowly leveled thereafter at  $28.3 \pm 2.5$  beats min<sup>-1</sup> (Fig. 5).

A small but significant change in  $\dot{Q}$  and power output of the heart occurred at a heart rate of 30 beats min<sup>-1</sup> in comparison to 60 beats min<sup>-1</sup> (control) (Fig. 6).  $\dot{Q}$  decreased from  $29.4 \pm 0.2$  ml min<sup>-1</sup> kg<sup>-1</sup> ( $N=7$ ) to  $26.8 \pm 0.5$  ml min<sup>-1</sup> kg<sup>-1</sup> and power output decreased from  $2.74 \pm 0.21$  mW g<sup>-1</sup> to  $2.49 \pm 0.17$  mW g<sup>-1</sup> at  $f_H=30$  beats min<sup>-1</sup> and  $f_H=60$  beats min<sup>-1</sup>, respectively. At the same time,  $P_{in}$  increased significantly from  $0.000 \pm 0.007$  kPa ( $N=7$ ) to  $0.053 \pm 0.013$  kPa and  $V_s$  increased from  $0.49 \pm 0.00$  ml kg<sup>-1</sup> to  $0.88 \pm 0.02$  ml kg<sup>-1</sup> (Fig. 6).

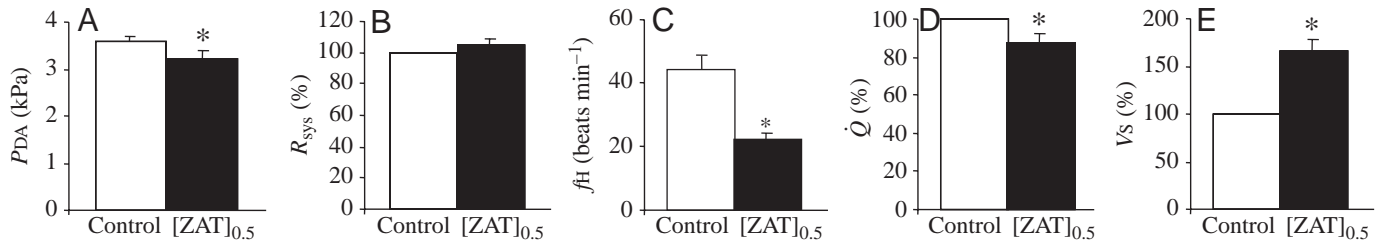


Fig. 3. Effect of zatebradine (ZAT) (dose equivalent to the  $K_m$  for each individual fish; see Materials and Methods) on (A) dorsal aortic pressure ( $P_{DA}$ ), (B) peripheral resistance ( $R_{sys}$ ), (C) heart rate ( $f_H$ ), (D) relative cardiac output ( $\dot{Q}$ ) and (E) relative stroke volume ( $V_s$ ). Values are means  $\pm$  S.E.M. ( $N=14$ ). Asterisks indicate significant difference from the control value ( $P<0.05$ ).

### Discussion

The results of the study indicate that zatebradine is a useful cardiac chronotropic molecule with which to manipulate heart rate in fish, as previously shown in mammals. Direct evidence of the link between heart rate and venous pressure is also presented. Lower heart rates attained by zatebradine injection cause an increased pressure in the sinus venosus that, in turn, increases stroke volume through the Frank–Starling mechanism. Such compensation between heart rate and stroke volume contributes to the short-term modulation of cardiac output *in vivo*.

#### *Zatebradine is a suitable bradycardic agent*

Zatebradine, a bradycardic agent, was tested as a means of manipulating heart rate within its physiological range (Altimiras and Larsen, 2000). Zatebradine-induced bradycardia occurs as a result of the inhibition of the

hyperpolarization-activated current in pacemaker cells, as shown in different mammalian species (Kobinger and Lillie, 1984; Schipke et al., 1991).

The suitability of zatebradine as a chronotropic agent also requires that it has no inotropic effects and no effect on peripheral resistance. As shown in Fig. 2, a dose of zatebradine of  $2.79 \pm 0.47 \text{ mg l}^{-1}$  decreased heart rate by half the maximum change, which compares well with similar experiments in mammals (Franke et al., 1987; Kalman et al., 1995; Ryu et al., 1996; Schipke et al., 1991). Thus, zatebradine is also a bradycardic agent in trout, indicating that the hyperpolarization current ( $I_f$ ) is also a component of the pacemaker currents in nodal cells in this species.

The results from the Series II experiments indicate that zatebradine has no significant effect on the peripheral vasculature because systemic resistance is unchanged (Fig. 3). The significant decrease in dorsal aortic pressure is due to the concomitant decrease in cardiac output when heart rate decreases to half the maximum effect (Fig. 3).

Finally, no significant inotropic effects of zatebradine were

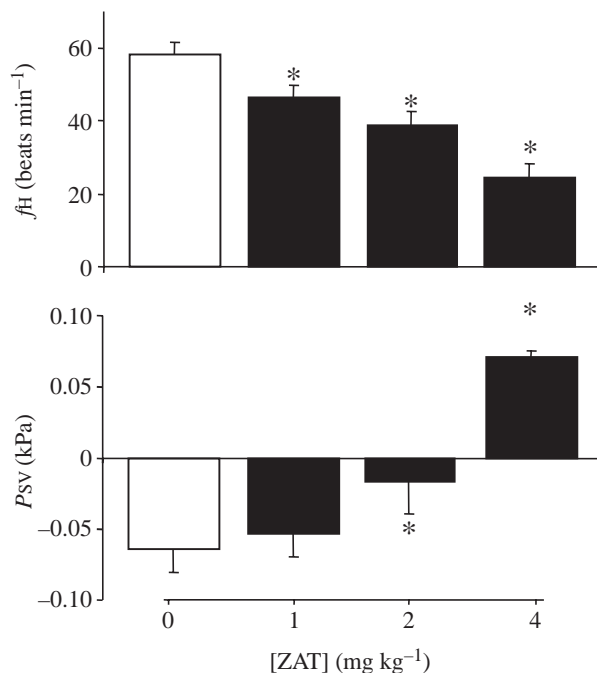


Fig. 4. Effect of different doses of zatebradine on heart rate and venous pressure *in vivo*. Values are means  $\pm$  S.E.M. ( $N=10$ ). Asterisks indicate significant difference from the control value ( $P<0.05$ ).

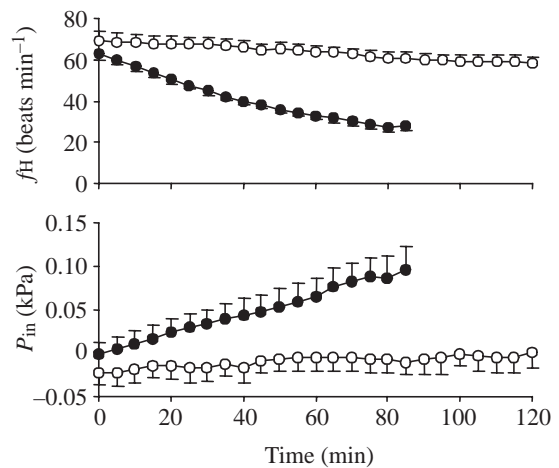


Fig. 5. Time-dependent effects of zatebradine ( $1 \text{ mg ml}^{-1}$ ) on heart rate ( $f_H$ ) and preload pressure ( $P_{in}$ ) in the perfused heart. Zatebradine-perfused hearts (closed symbols,  $N=5$ ) are shown together with control perfused hearts (open symbols,  $N=4$ ) to account for the possible deterioration of the preparation over time. Values are means  $\pm$  S.E.M.

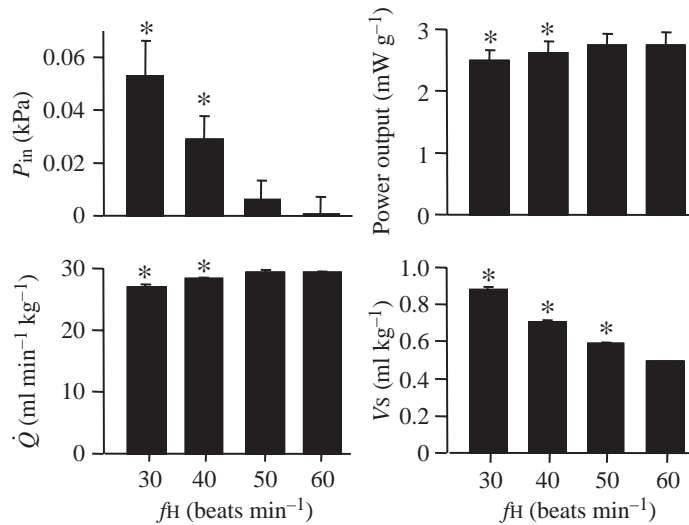


Fig. 6. Relationship between heart rate ( $f_H$ ) and preload pressure ( $P_{in}$ ), cardiac output ( $\dot{Q}$ ), power output of the heart ( $\text{mW g}^{-1}$ ) and stroke volume ( $V_s$ ) in the hearts perfused with Ringer saline and zatebradine ( $1 \text{ mg ml}^{-1}$ ). Values are means  $\pm$  S.E.M. ( $N=5$ ). Asterisks indicate significant difference from the control value (at  $60 \text{ beats min}^{-1}$ ) ( $P<0.05$ ).

found in isolated ventricular strips (Table 1), as was the case for mammals (Chen and Slinker, 1992).

Altogether these results indicate that zatebradine is an appropriate pharmacological tool to manipulate heart rate without other significant cardiovascular changes and as such, is well suited to study both *in vivo* and *in vitro* how heart rate affects intrinsic cardiac regulation.

#### Pressure in the sinus venosus and mechanisms of atrial filling

Pressure in the sinus venosus in control conditions was negative in seven out of ten fish, with a mean value of  $-0.06 \pm 0.04 \text{ kPa}$  (Fig. 4). To our knowledge these are the first pressure measurements in the sinus venosus of a teleost fish and directly corroborate the predictions based on data from perfused hearts (Farrell and Jones, 1992). Values for central venous pressure in trout obtained in other studies indicate values slightly above ambient, averaging  $0.37 \text{ kPa}$  in the Cuvierian ducts (at an average heart rate of  $62.8 \text{ beats min}^{-1}$  at  $12^\circ\text{C}$ ; Olson et al., 1997) or  $0.19 \text{ kPa}$  in the common cardinal vein (at an average heart rate of  $32 \text{ beats min}^{-1}$  at  $10^\circ\text{C}$ ; Kiceniuk and Jones, 1977). Collectively, these values portray a pressure gradient from slightly above ambient in the central veins to slightly subambient in the sinus venosus that could support the operation of the *vis-à-fronte* mechanism for atrial filling (Farrell and Jones, 1992).

*Vis-à-fronte* filling is allegedly associated with resting conditions and low stroke volumes in active teleosts because filling pressures above ambient (*vis-à-tergo* filling) are required for normal and elevated stroke volumes (Farrell and Jones, 1992). In resting conditions, however, heart rates are considerably lower. Recent studies in trout using surgery-free methods indicate that true resting heart rates at  $15^\circ\text{C}$  are in the

order of  $30 \text{ beats min}^{-1}$  (Altimiras and Larsen, 2000), well below the control  $f_H$  value obtained in this study ( $58.3 \pm 3.5 \text{ beats min}^{-1}$ ). Such low heart rates could not be attained in surgically instrumented animals, so zatebradine was used instead. Lowering the heart rate lengthens the filling time and  $P_{sv}$  increases ( $0.07 \pm 0.05 \text{ kPa}$  at  $f_H = 24.6 \pm 3.7 \text{ beats min}^{-1}$ ; Fig. 4), which questions the existence of negative pressures in the sinus venosus in resting conditions and, at the same time, casts doubts on the *in vivo* relevance of *vis-à-fronte* atrial filling. Minerick et al. (2003) have recently reached the same conclusion by demonstrating a dynamic coupling between venous pressure and cardiac output.

#### Role of heart rate in the regulation of cardiac output

The rise of  $P_{sv}$  associated to the progressive decrease in heart rate from the Series III experiments also provided a mechanistic explanation of the autoregulation of cardiac output observed *in vivo*. Thus, the small decrease in  $\dot{Q}$  down to  $87.5 \pm 4.6\%$  when  $f_H$  was halved with zatebradine would be explained by a rise in  $P_{sv}$  that would allow a concomitant increase in  $V_s$  to  $165 \pm 13\%$  (Fig. 3), according to the Frank–Starling relationship. Complete compensation was not attained, perhaps because maximal stroke volume was reached (Forster and Farrell, 1994).

The mechanism of cardiac output autoregulation related to variations in heart rate and filling pressure (denominated time-dependent autoregulation in the rest of the Discussion) was further verified in perfused hearts with the simultaneous measurement of  $V_s$  and  $P_{in}$  at varying heart rates. As shown in Fig. 6, the zatebradine-mediated drop in heart rate is coupled to a simultaneous increase in  $P_{in}$  and  $V_s$  similar in magnitude to the *in vivo* values.

Time-dependent autoregulation enhances the repertoire of intrinsic cardiac regulation to include the effects of varying filling time. Strictly speaking, the mechanism is dependent on the extrinsic neurohumoral modulation of heart rate, but its compensatory role is exerted through changes in stroke volume by riding on the Starling curve of the heart. The impact of time-dependent autoregulation, however, is limited by the interaction with other mechanisms regulating venous pressure and stroke volume. Adrenaline, for instance, is known to increase venous pressure (Zhang et al., 1998).

At high heart rates, the low venous pressures expected from short filling times are likely to be counteracted by the positive inotropic effect related to adrenergic activation. This prediction needs to be confirmed experimentally, but it is already known that energetically demanding conditions such as exercise require elevated cardiac outputs achieved by increasing heart rate and stroke volume simultaneously, and this is incompatible with subambient or low venous pressures (Farrell et al., 1996). In the absence of adrenergic stimulation, a reciprocal relationship between  $f_H$  and  $V_s$  has already been shown in paced trout hearts *in vitro* (Farrell et al., 1989). Besides the possibility that short filling times limit stroke volume, a reduced force of contraction resulting from the negative staircase effect was also speculated (Farrell and Jones, 1992).

At low heart rates, a longer filling time promotes an increase in venous pressure that results in atrial distension. In turn, atrial stretch triggers the release of atrial natriuretic factor (ANF) (Cousins and Farrell, 1996), which might break down the reciprocal  $f_H$ - $V_S$  relationship because ANF is a potent vasodilator that lowers venous pressure (Olson et al., 1997).

Such a cardioprotective role of ANF (Farrell and Olson, 2000) is not incompatible with the time-dependent autoregulatory mechanism proposed in this study, which is targeted at a more physiological range of sinus venous pressures. ANF release in freshwater- and seawater-acclimated perfused trout hearts is evident at filling pressures above 0.1 kPa (Cousins et al., 1997), while the results presented indicate the operation of the time-dependent autoregulatory mechanism from subambient to pressures slightly below 0.1 kPa.

#### List of symbols

$f_H$	heart rate
$M_V$	ventricular mass
$P_{DA}$	pressure in dorsal aorta
$P_{in}$	preload pressure
$P_{out}$	afterload pressure
$P_{SV}$	pressure in <i>sinus venosus</i>
$\dot{Q}$	cardiac output
$R_{sys}$	systemic vascular resistance
$V_S$	stroke volume

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