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# Modulatory effects of adenosine and adenine nucleotides on different heart preparations of the American lobster, *Homarus americanus*

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## SUMMARY

**As shown previously, adenosine and the adenine nucleotides cause a rapid increase in heart rate ($f_H$) and haemolymph velocity ($v_{HL}$) when infused into intact American lobster (*Homarus americanus*). Here we compare the effects of adenosine and adenine nucleotides on different heart preparations in order to gain insight into their sites of action. In the semi-isolated (*in situ*) heart preparation where the heart is uncoupled from neural and hormonal influence AMP, ADP and ATP, but not adenosine increased contractile force. None of the purines altered $f_H$. Thus, the adenine nucleotides directly affect the myocardium and not the $f_H$-setting cardiac ganglion.**

**In cardioregulatory-denervated animals in which the cardiac ganglion only was severed from the central nervous system (CNS), purines caused a small and gradual increase in $f_H$, indicating that *in vivo* an alteration of $f_H$ arises indirectly through the central nervous system which in turn sends the information to the heart *via* the dorsal nerves. The gradual increase in $f_H$ of cardioregulatory-denervated animals may also result from neurohormones released into the circulatory system, although no significant changes in haemolymph concentration of dopamine, serotonin and octopamine were found during adenosine infusion.**

**In semi-isolated (*in situ*) hearts adenine nucleotides also increased haemolymph flow, as a consequence of increased heart contractile force, but again adenosine had no effect. These data show that *in vivo* adenosine does not influence the myocardium, only the adenine nucleotides affect the myocardium directly. Obviously adenosine possesses an indirect effect, perhaps on cardio-arterial valves and arterial resistance, but other, as yet unidentified, modifying factors are also possible.**

Key words: adenosine, adenine nucleotides, heart rate, haemolymph velocity, lobster, *Homarus americanus*.

## INTRODUCTION

In the open circulatory system of the American lobster, *Homarus americanus*, haemolymph flow is driven throughout the body by a semi-autonomous, neurogenic heart *via* seven main arteries (Maynard, 1960). The heart consists of a single ventricle suspended in the pericardial sinus by an array of alary ligaments. The ligaments are stretched during systole and the stored energy is used to re-expand the heart during diastole and causes refilling of the heart with haemolymph through three pairs of valved ostia (McMahon and Burnett, 1990; McMahon, 1995).

The basic heart rhythm arises from the cardiac ganglion located at the inner dorsal wall of the heart (Alexandrowicz, 1932). Modification of this main rhythm is controlled by different factors, such as the cardioregulatory nerves arising from the subesophageal ganglion and by cardioactive neurohormones. Each nerve contains two accelerator axons and one inhibitory axon (Alexandrowicz, 1932). In the crayfish, *Procambarus clarkii*, stimulation of the cardioaccelerator neurons increases heart rate ($f_H$) and contractility. By contrast, stimulation of the cardioinhibitory neurons causes bradycardia or even cardiac arrest (Wilkens and Walker, 1992).

Cardioactive neurohormones are released from the pericardial organs (POs) situated in the pericardial sinus adjacent to the heart. These include the amines dopamine (DA), serotonin (5-HT) and octopamine (OA) as well as the peptides proctolin (PR), crustacean cardioactive peptide (CCAP) and FMRFamide-like peptides (Cooke and Sullivan, 1982; Stangier et al., 1986; Stangier et al., 1987; Trimmer et al., 1987). Cardioexcitatory effects of neurohormones on semi-isolated (*in situ*) hearts of decapod crustacean have been described previously (Saver and Wilkens, 1998; Saver et al., 1998; Wilkens and McMahon, 1992; Wilkens and Mercier, 1993; Wilkens et al., 2005). Modulatory effects on cardiac performance resulting from the infusion of neurohormones also occur *in vivo*, but the effects are less dramatic than on semi-isolated (*in situ*) hearts (Wilkens et al., 1985; McGaw et al., 1994; McGaw et al., 1995; Guirguis and Wilkens, 1995).

Neurohormones can also act on the cardioarterial valves located at the origin of each artery, except the posterior aorta (Kuramoto and Ebara, 1984; Kuramoto et al., 1992; Kuramoto et al., 1995; Wilkens and Kuramoto, 1998) and directly on the arteries (Wilkens, 1997; Wilkens and Taylor, 2003). Thus, these effectors regulate not only cardiac activity, but also the distribution of haemolymph in response to different physiological situations, such as oxygen deficiency, locomotion and feeding (McMahon and Wilkens, 1975; McMahon, 1992; Airriess and McMahon, 1994; DeWachter and McMahon, 1996; Reiber and McMahon, 1998; Rose et al., 1998; Rose et al., 2001; McGaw, 2004). The effectors might help to prevent an oxygen debt or to compensate for an existing oxygen debt.

Additional metabolic factors such as L-lactate and urate (Truchot, 1980; Morris et al., 1985; Bridges and Morris, 1986; Lallier et al., 1987; Zeis et al., 1992), accumulating in the haemolymph during functional or environmental anaerobiosis, bind

to hemocyanin and increase the oxygen affinity of the pigment, thus enhancing oxygen delivery to the tissues (for details, see Grieshaber et al., 1994). Adenosine, AMP, ADP, ATP are additional metabolic factors serving *in vivo* to compensate for an oxygen deficiency by increasing $f_H$, scaphognathite frequency and haemolymph velocity ($v_{HL}$) (Stegen and Grieshaber, 2001). During extensive tail flipping of the lobster the adenosine content increases significantly in the abdominal muscle, whereas only a slight accumulation can be detected in the haemolymph. However, a transient build up of adenosine in the haemolymph can be assumed, because a pronounced accumulation of inosine occurred. Adenosine is obviously channeled into the haemolymph where it is rapidly degraded to inosine through the action of a potent adenosine deaminase (E. Stegen, G.M. and M.K.G., unpublished). These data imply that adenosine and the adenine nucleotides may be responsible for an increased haemolymph flow observed in intact animals during hypoxic conditions.

It is still not known how adenosine and adenine nucleotides mediate their effects on the cardiovascular system *in vivo*. To investigate the modulatory effects of adenosine and the adenine nucleotides on the heart, we used the semi-isolated (*in situ*) heart preparation similar to that of Wilkens and Mercier (Wilkens and Mercier, 1993), in which the heart is uncoupled from neural as well hormonal control, and the *in vivo* heart preparation, in which the cardioregulatory dorsal nerves only are cut (Guirguis and Wilkens, 1995). The results from these two different preparations should allow us to decide whether adenosine and its nucleotides exert a direct effect on the heart or whether their effects are indirect, i.e. act on the CNS, which in turn controls the cardioregulatory nerves and the release of neurohormones into the haemolymph.

## MATERIALS AND METHODS

### Semi-isolated (*in situ*) heart preparation

Experiments with semi-isolated (*in situ*) heart preparations were performed at the Department of Biological Science, University of Calgary, Calgary. *Homarus americanus* (Milne-Edwards, 1837) weighing between 500 and 600 g were purchased from a local supplier. Animals were used immediately or held up to a week in recirculating artificial seawater at 12±2°C and a salinity of 32±2‰ prior to the experiments. Lobsters were not fed during that time.

Preparation of the semi-isolated (*in situ*) heart was performed according to Wilkens and Mercier (Wilkens and Mercier, 1993). Animals were anaesthetized on crushed ice for 20 min. After the brain was destroyed, the abdomen and the ventral part of the animal were cut away, obtaining the dorsal part of the carapace containing the heart. The heart was rinsed with saline and all tissues overlaying the heart were removed. The carapace was then pinned ventral side up into a 150 ml organ bath filled with saline at a temperature of 12±1°C. The saline consisted of 530 mmol $l^{-1}$ NaCl, 10.7 mmol $l^{-1}$ KCl, 18 mmol $l^{-1}$ $CaCl_2.2H_2O$, 24.6 mmol $l^{-1}$ $MgCl_2.6H_2O$, 3 mmol $l^{-1}$ Tris Base® pH 7.6.

The posterior aorta was cannulated with a polyethylene tube (2.27 mm ID), tip size similar to vessel diameter (1.86 mm; mean diameter) (Reiber et al., 1997). Because all arteries were tied off with a 1.0 surgical silk thread the flow was directed through the cannula which was connected to an in-line Flow Transducer (Type 2, 3.2 mm ID) coupled to a T106 Animal Research Flowmeter (Transonic Inc., Ithaca, USA).

The heart was continuously perfused with saline at 2 ml $min^{-1}$ through a cannula inserted into the heart. Adenosine, AMP, ADP and ATP dissolved in saline were also administered through this cannula by switching from saline to purine solution. Ventricular pressure was measured *via* a cannula inserted through the ventral wall of the heart connected to a pressure transducer (Model P23Db, Gould Statham, Oxnard, USA). Since all cardiac suspensory ligaments and the pericardial septum remained intact, the heart aspirates saline through the valved ostia and beats naturally for several hours. A possible direct influence of purines on $f_H$, cardiac output, ventricular pressure and stroke volume can be revealed since the central origins of the neurosecretory neurons of the cardioregulatory and pericardial organs (POs) are severed. Each experiment was divided into a control period lasting for 5 min, a perfusion period of 1 min, and a recovery period of 19 min.

### *In vivo* pulsed-Doppler flowmetry

Doppler experiments were conducted at the Institut für Zoophysiologie, Heinrich-Heine-Universität, Düsseldorf. Specimens of *Homarus americanus* were purchased from a local supplier and were kept in seawater at 15±2°C and a salinity of 35±1‰. Animals used for Doppler experiments were fed with frozen squid once a week.

The $f_H$ and $v_{HL}$ in the sternal artery, posterior aorta and left lateral artery were measured by means of a pulsed-Doppler flow meter (545C-4 Bioengineering, University of Iowa, USA) (Reiber et al., 1992). Measurements were taken from intact animals and animals in which the cardioregulatory nerves were cut.

To obtain cardioregulatory-impaired animals, denervation surgery was performed according to Guirguis and Wilkens (Guirguis and Wilkens, 1995). Animals were anaesthetized on crushed ice for 20 min. Next a square of the carapace and the hypodermis dorsal to the heart were removed. To prevent loss of haemolymph a dam molded from modeling clay was placed around the opening. The cardioregulatory nerves approaching the dorsal surface of the heart were cut by making a superficial transection through the connective tissue, close to the posterior ligament. The carapace square was replaced and sealed with dental wax and cyanoacrylate adhesive. Animals were allowed to recover for more than 12 h before tests were conducted. Successful transection of the cardioregulatory nerves was confirmed by the loss of the so-called startle response. These periods of bradycardia occur in settled lobsters when an unseen stimulus happens, for instance when shadows sweep over the animal's visual field or when the carapace is touched. Sham operations consisted of removing the square of carapace and opening the hypodermis only. Preparations complied with the guidelines of the German animal protection law (§8a, TSchG; AZ. 50.05-240-114/05, –33/05, –31/04).

In these *in vivo* preparations adenosine, AMP, ADP and ATP solutions dissolved in lobster saline, pH 8.0 (Zeis et al., 1992) were administered for 10 min into the infrabranchial sinus at the base of the fourth pereiopod by means of a syringe pump (Infusor, Basel, Switzerland). All infusions were carried out with a solute concentration calculated to the body mass of the animal. The same infusion speed was used for all experiments, resulting in an equal solute concentration within the animals. Control experiments were accomplished by infusing Ringer solution into the infrabranchial sinus.

### Estimation of the concentration of DA, OA and 5-HT

To estimate the concentration of DA, OA and 5-HT in the haemolymph during the infusion of adenosine, haemolymph samples were drawn from the pericardial sinus. Blood samples, up to a maximum volume of 1 ml were collected through a perforation drilled into the carapace prior to the experiments. Haemolymph samples were centrifuged for 5 min at 2500 ***g*** at 4°C. Dopamine

and 5-HT samples were diluted (1:2 v/v) with Ringer solution and isoproterenol was added as internal standard at a final concentration of 50 nmol $l^{-1}$. Samples were centrifuged for 30 min at 5000 ***g*** in Ultrafree®-MC Centrifugal Filter Units (Millipore, Bedford, USA) according to the method of Wood and Hall (Wood and Hall, 2000), to remove protein with a cut off ⩾10 kDa. The eluate was directly used to estimate DA and 5-HT by high pressure liquid chromatography (L-6200A Intelligent Pump, LS-7200 LaChrom Autosampler, Merck/Hitachi, Darmstadt/Tokio, Germany and a Pulse Damper, Model 5563, SSI-ESA, Bedford, Massachusetts, USA) equipped with a Purospher®STAR RP-18 column (150 4.6 mm, 5 μm; VWR, Darmstadt, Germany) and an electrochemical detector (Model 5100A Coulochem II Multi-Electrode Electrochemical Detector with High-sensitivity Analytical Cell, Model 5011, ESA, Bedford, Massachusetts, USA.). Compounds were separated isocratically at a flow of 0.75 ml $min^{-1}$ at 18°C. The eluent consisted of 0.75 mmol $l^{-1}$ $NaH_2PO_4$, 2.5 mmol $l^{-1}$ heptane-sulfonic acid, 20 μmol $l^{-1}$ EDTA and 25% methanol, pH 5.6. To oxidize DA and 5-HT, the potential of the coulometric electrode was set at 50 mV and the amperometric electrode at 300 mV, which allowed detection of DA and 5-HT with a limit of 1 nmol $l^{-1}$ and 0.5 nmol $l^{-1}$, respectively (ESA, Coulochem Applications, Bedford, MA, USA).

To estimate OA, an aliquot of haemolymph was mixed with ice-cold 0.1 mmol $l^{-1}$ perchloric acid (1:5 v/v) and centrifuged for 20 min at 25 000 ***g***. The supernatant was analyzed by EC-HPLC (LC-125 Solvent Modules, LC-507e Autosampler, Beckmann Instruments, Inc. USA) using a Purospher®STAR RP-18 column (150 4.6 mm, 5 μm). The potential of the electrochemical detector (Bioquant PAM2, Bischoff, Leonberg, Germany) was set at 750 mV. Octopamine was eluted isocratically at 0.5 ml $min^{-1}$ using a solvent containing 0.75 mmol $l^{-1}$ $NaH_2PO_4$, 3 mmol $l^{-1}$ heptane-sulfonic acid, 20 μmol $l^{-1}$ EDTA and 15% methanol, pH 3.0 at 30°C (ESA, Coulochem Applications, Bedford, MA, USA). Under these conditions a detection limit of 2 nmol $l^{-1}$ was achieved.

Data in this study are presented as means ± s.d. To compare control values with those obtained during and after the different interventions, a one way repeated measures ANOVA was performed on raw data. Multiple comparisons *versus* control values were carried out using the Holm–Sidak test. Differences were considered significant at $P<0.05$.

## RESULTS

### Semi-isolated (*in situ*) heart preparation

After reaching a stable baseline the semi-isolated (*in situ*) hearts were perfused with solutions containing 2.5 mmol $l^{-1}$ each of adenosine, AMP, ADP or ATP for 1 min and the effects on $f_H$, cardiac output ($\dot{V}_b$), ventricular pressure ($P_{vent}$) and stroke volume ($V_S$) were recorded (Fig. 1). None of the drugs caused a significant change in $f_H$, although ATP caused a transient bradycardia.

The $\dot{V}_b$ was not affected significantly by adenosine perfusion. By contrast, perfusion with adenine nucleotides raised the $\dot{V}_b$ in the order of AMP, ADP and ATP, with ADP and ATP causing significant increases. Adenosine and the adenine nucleotides produced similar changes in $P_{vent}$ and $V_S$ as in $\dot{V}_b$ (Table 1).

### *In vivo* measurements using the pulsed-Doppler flowmetry

The $f_H$ and $v_{HL}$ were measured in the posterior aorta, sternal artery and left lateral artery of intact and cardioregulatory-denervated animals. In the latter, heart activity is not affected by the central nervous system, but hormonal regulation can still persist. In intact animals both effectors are present and thus allow a direct comparison of the regulatory effects of adenosine and adenine nucleotides infused into both preparations.

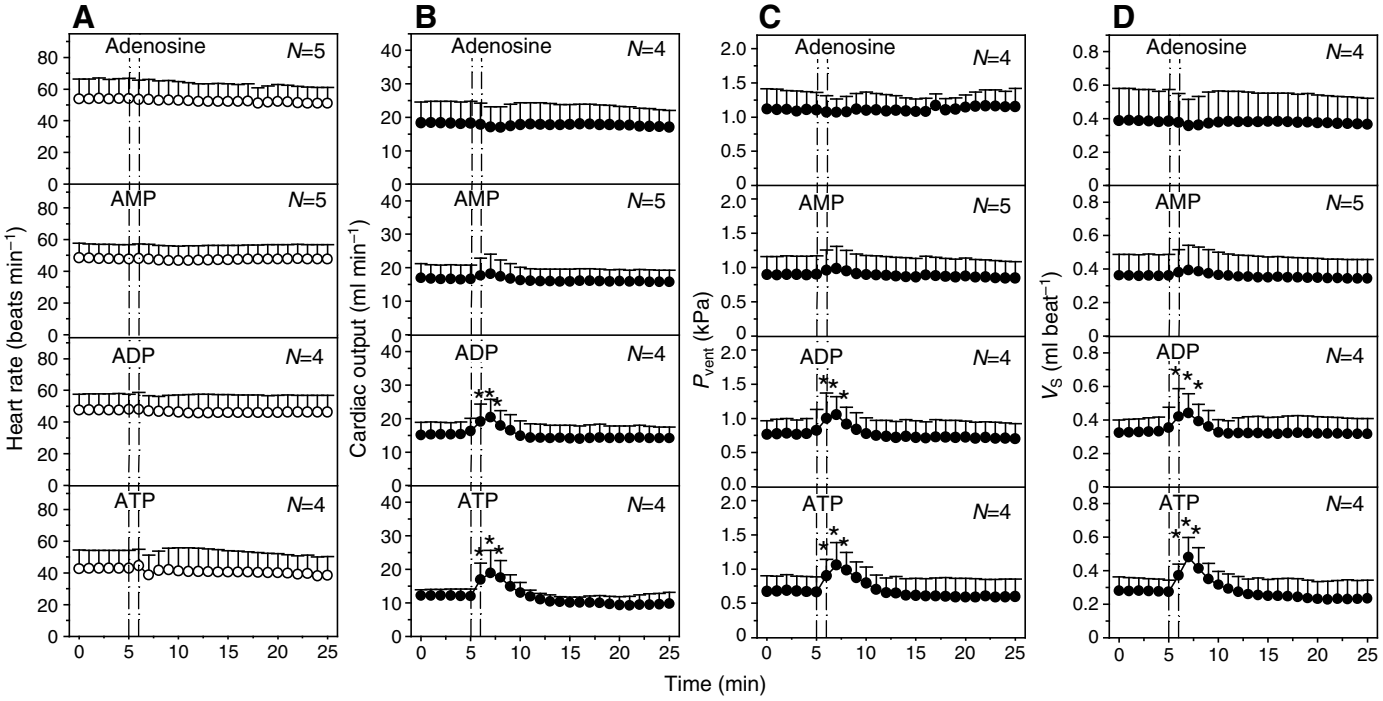


Fig. 1. Comparison of (A) heart rate (beats $min^{-1}$), (B) cardiac output (ml $min^{-1}$), (C) ventricular pressure (kPa) and (D) stroke volume (ml $beat^{-1}$) in semi-isolated hearts of the American lobster, *Homarus americanus*, before ($t_0$–$t_5$), during ($t_6$–$t_7$) and after ($t_8$–$t_{25}$) the perfusion of 2.5 mmol $l^{-1}$ solutions of adenosine or adenine nucleotides. The vertical dashed lines indicate the infusion period. Data are presented as means ± s.d. (*N* is given in the respective graphs). An asterisk denotes a value significantly different from the control value ($P\leqslant0.05$).

Table 1. Effects of adenosine and adenine nucleotide perfusion on heart rate, cardiac output, ventricular pressure and stroke volume of semi-isolated hearts

| | Adenosine (2.5 mmol $l^{-1}$) | | AMP (2.5 mmol $l^{-1}$) | | ADP (2.5 mmol $l^{-1}$) | | ATP (2.5 mmol $l^{-1}$) | |
|---|---|---|---|---|---|---|---|---|
| | Control | $E_{max}$ | Control | $E_{max}$ | Control | $E_{max}$ | Control | $E_{max}$ |
| $f_H$ | 54±13 | 51±10 | 48±9 | – | 48±10 | – | 46±11 | 41±12 |
| $\dot{V}_b$ | 18.3±6.4 | 16.7±6.0 | 16.7±4.2 | 18.6±5.5 | 15.4±3.7 | 20.9±5.4 | 12.1±1.7 | 19.9±7.4 |
| $P_{vent}$ | 1.11±0.28 | 1.04±0.17 | 0.9±0.27 | 1.0±0.24 | 0.78±0.23 | 1.1±0.33 | 0.63±0.21 | 1.09±0.28 |
| $V_S$ | 0.39±0.19 | 0.36±0.16 | 0.36±1.3 | 0.4±0.14 | 0.33±0.09 | 0.46±0.15 | 0.28±0.08 | 0.48±0.12 |

$f_H$, heart rate (beats $min^{-1}$); $\dot{V}_b$, cardiac output (ml $min^{-1}$); $P_{vent}$, ventricular pressure (kPa); $V_S$, stroke volume (ml $beat^{-1}$).
Given are the mean value of the control period (control) and the maximal value reached ($E_{max}$). Data are presented as means ± s.d. ($N$=5 adenosine and AMP and 4 ADP and ATP).

### Adenosine- and adenine nucleotide infusion

When infused *in vivo* into the infrabranchial sinus, adenosine and adenine nucleotides were found to increase $f_H$ and $v_{HL}$ in the American lobster (Stegen and Grieshaber, 2001). Hence, the effects of adenosine, AMP, ADP and ATP at a concentration of 2.4 nmol $min^{-1}$ $g^{-1}$ body mass were compared in intact lobsters and in animals in which the cardioregulatory nerves were cut.

In Fig. 2 the $f_H$ of intact and cardioregulatory-denervated lobsters are compared before ($t_0$–$t_{10}$), during ($t_{11}$–$t_{20}$) and after the infusion of adenosine and adenine nucleotides ($t_{21}$–$t_{60}$) at a concentration of 2.4 nmol $min^{-1}$ $g^{-1}$ body mass. In intact, resting lobsters all drugs caused a rapidly and significantly increased $f_H$. The $f_H$ remained elevated during the recovery period and was protracted in the order AMP<ADP<ATP. The $f_H$ during the control periods was similar to that previously published (Wilkens et al., 1996; Wilkens and Kuramoto, 1998).

In cardiac-denervated animals all drugs caused a gradual increase in $f_H$, but these were significant only for AMP and ATP. A comparison of the $f_H$ after 10 min of infusion (Fig. 3; $t_{20}$) in the two preparations demonstrated that this parameter was more pronounced in intact than in cardioregulatory-denervated animals.

The $f_H$ of sham-operated animals, in which a square of the carapace was removed and replaced after opening the hypodermis, leaving the cardioregulatory nerves intact, is presented in Fig. 4. Here, adenosine infusion caused a significant increase in $f_H$ in the same way as in intact animals.

No effects on $f_H$ and on $v_{HL}$ were observed in either preparation when Ringer solution was administered into the circulatory system (Fig. 5).

As opposed to $f_H$, cardiac denervation did not alter the time course of drug-induced changes in haemolymph velocity. The changes in $v_{HL}$ in the posterior aorta, the sternal artery and the left

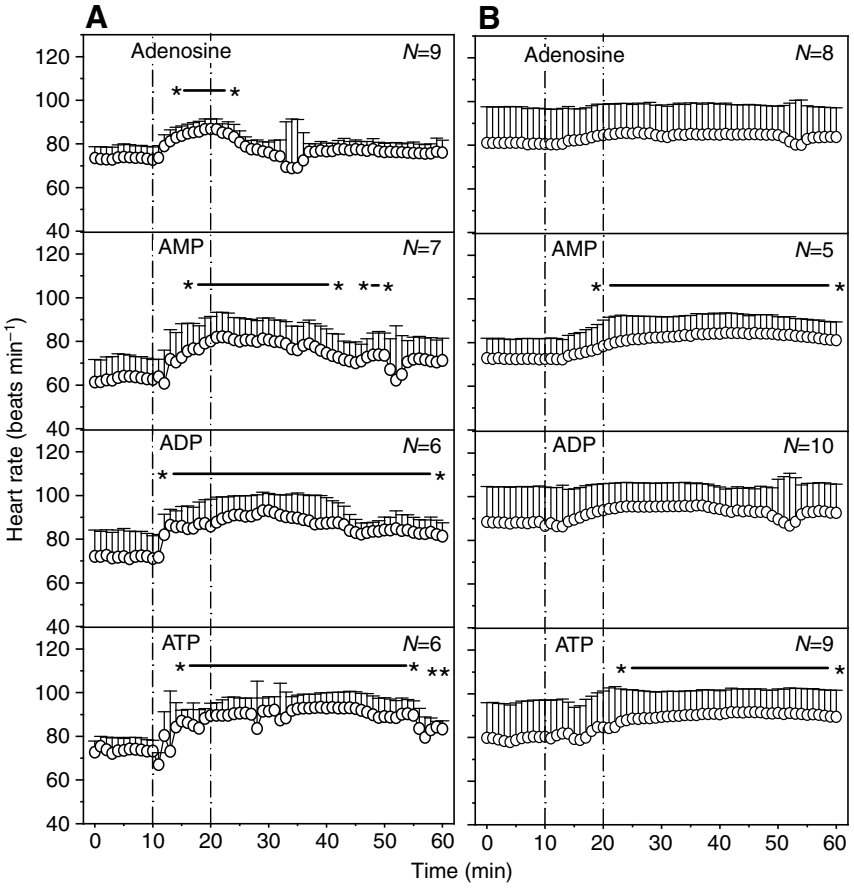


Fig. 2. Comparison of heart rate (beats $min^{-1}$) in intact (A) and cardioregulatory-denervated (B) *Homarus americanus* before ($t_0$–$t_{10}$), during ($t_{11}$–$t_{20}$) and after ($t_{21}$–$t_{60}$) the perfusion of a solution of 2.4 nmol $min^{-1}$ $g^{-1}$ body mass of adenosine or adenine nucleotides. The vertical dashed lines indicate the infusion period. Data are presented as means ± s.d. ($N$ is given in the respective graphs). All values under the lines with asterisks at each end are significantly different from the control value ($P \leqslant 0.05$).

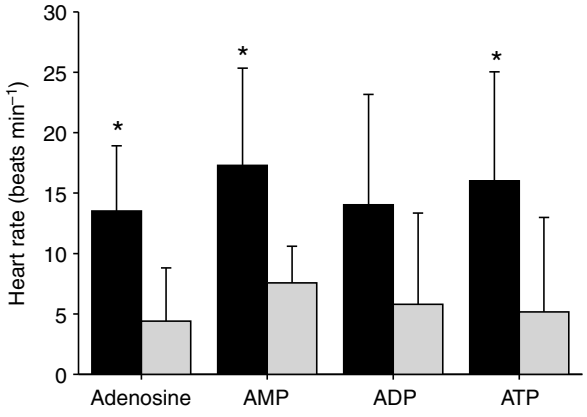


Fig. 3. Comparison of heart rate in intact (black bars) and cardioregulatory-denervated (grey bars) *Homarus americanus*. Values are the differences between the mean value of the control period and the value at $t_{20}$ during the perfusion of a solution of 2.4 nmol min$^{-1}$ g$^{-1}$ body mass of adenosine or adenine nucleotides. $t_{20}$ corresponds with the end of the infusion. Data are presented as means ± s.d. An asterisk denotes a value significantly different from the control value ($P \leqslant 0.05$). [$N$=9 (intact) and 10 (cardioregulatory denervated) for adenosine; 7 and 5 for AMP; 6 and 0 for ADP, and 6 and 9 for ATP.]

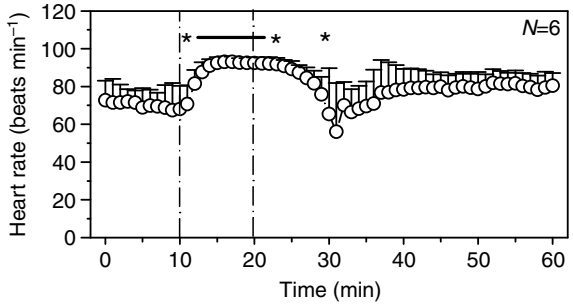


Fig. 4. Heart rate (beats min$^{-1}$) of sham operated lobsters before ($t_0$–$t_{10}$), during ($t_{11}$–$t_{20}$) and after ($t_{21}$–$t_{60}$) the perfusion of a solution of 2.4 nmol min$^{-1}$ g$^{-1}$ body mass of adenosine. The vertical dashed lines indicate the infusion period. Data are presented as means ± s.d. All values under the line with asterisks at each end are significantly different from the control value ($P \leqslant 0.05$) ($N$ is given in the respective graphs).

lateral artery during drug perfusion where similar in magnitude and time course for intact and denervated animals and the changes were significant in each case (Figs 6–8; for values see Table 2).

In intact animals, $v_{HL}$ in the posterior aorta was maintained at elevated levels during the recovery period after perfusion in the order adenosine<AMP<ADP<ATP, whereas in cardioregulatory-denervated animals there was no correlation between the duration of the effects and the infused purines (Fig. 6). In the sternal artery $v_{HL}$ was similarly maintained at elevated levels as in the posterior aorta. In cardioregulatory-denervated animals the effect of the infused purines was in the order adenosine<ADP<AMP<ATP (Fig. 7). In the lateral artery $v_{HL}$ showed the same time course as in the other arteries for all the purines with the exception of AMP. And in cardioregulatory-denervated lobsters there was no correlation between the duration of the effects and the infused purines (Fig. 8).

### Quantification of DA, 5-HT and OA

The gradual increase in $f_H$ and the mainly significant enhancement in haemolymph velocities in cardioregulatory-denervated animals could result from a release of neurohormones from the POs. To verify the possible influence of adenosine on the release of neurohormones, DA, 5-HT and OA were quantified in the haemolymph of the lobster (Fig. 9).

In one out of five animals, the DA concentration was below the detectable limit of 1 nmol l$^{-1}$. Under quiescent conditions ($t_0$) the DA concentration was 1.7±1.7 nmol l$^{-1}$. After the onset of adenosine infusion DA rose insignificantly to 2.2±1.4 nmol l$^{-1}$ at $t_{7.5}$ min and reached a maximum value of 2.2±1.5 nmol l$^{-1}$ at $t_{12,5}$. At the end of the recovery period ($t_{30}$) the concentration was 1.6±1.5 nmol l$^{-1}$.

In two out of five animals 5-HT was below the detectable limit of 0.5 nmol l$^{-1}$. The 5-HT was first detected at $t_{7.5}$ and $t_{10}$ in two other animals, respectively. In resting animals ($t_0$) a concentration of 0.1±0.2 nmol l$^{-1}$ was measured. At $t_5$ no 5-HT was detected for the reasons mentioned above. Starting at $t_{7.5}$ the 5-HT concentration increased insignificantly and reached 0.2±0.3 nmol l$^{-1}$at $t_{10}$ and 0.3±0.4 nmol l$^{-1}$ at $t_{15}$. At the end of the experiment ($t_{30}$) the calculated concentration was 0.1±0.3 nmol l$^{-1}$.

In one animal OA was detected only at $t_0$ and $t_{15}$. At the other timepoints OA was below the detectable limit of 2 nmol l$^{-1}$. With exception of $t_{2,5}$ (2.7±2.7 nmol l$^{-1}$) an approximately constant OA concentration was detected during the first 12.5 min of the infusion experiment. During the recovery period OA was insignificantly elevated.

Table 2. Effects of adenosine and adenine nucleotide infusion on haemolymph velocity in different vessels of intact and cardioregulatory-denervated *Homarus americanus*

| | Haemolymph velocity | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Adenosine* | | AMP* | | ADP* | | ATP* | |
| | Intact | Denervated | Intact | Denervated | Intact | Denervated | Intact | Denervated |
| Posterior aorta | 29.2±10.1 (98.9±43.2) | 41.9±19.8 (87.4±49.4) | 38.7±16.9 (92.9±25.8) | 39.3±11.8 (88.9±21.3) | 41.8±14.8 (115.3±23.5) | 29.8±5.5 (106.4±49.8) | 34.9±15.1 (98.3±42.8) | 34.2±11.9 (68.7±24) |
| Sternal artery | 35.3±8.8 (123.2±42.9) | 54.1±22.2 (147.7±67.6) | 67.7±19.5 (152±48.6) | 67.7±25.2 (157.8±45.2) | 88.1±26.5 (194.5±72.9) | 51.1±18.1 (106.4±49.8) | 79.8±23.4 (200.6±48.5) | 57.0±17.3 (121.7±35.8) |
| Lateral artery | 17.7±5.3 (54.3±20.7) | 31.1±14.1 (60.4±25.7) | 24.7±7.80 (61.7±35.8) | 52.7±25.3 (100.6±107.7) | 34.2±12.6 (94.2±27.5) | 41.6±16.5 (83.4±33.2) | 35.2±13.3 (115.1±63.1) | 46.1±31.4 (74.2±41.2) |

Haemolymph velocity ($v_{HL}$, mm min$^{-1}$).*2.4 nmol min$^{-1}$ g$^{-1}$ body mass. Given are the mean value of the control period and the maximal value reached (in parentheses). Data are presented as means ± s.d. (for $N$ values see Figs 6–8).

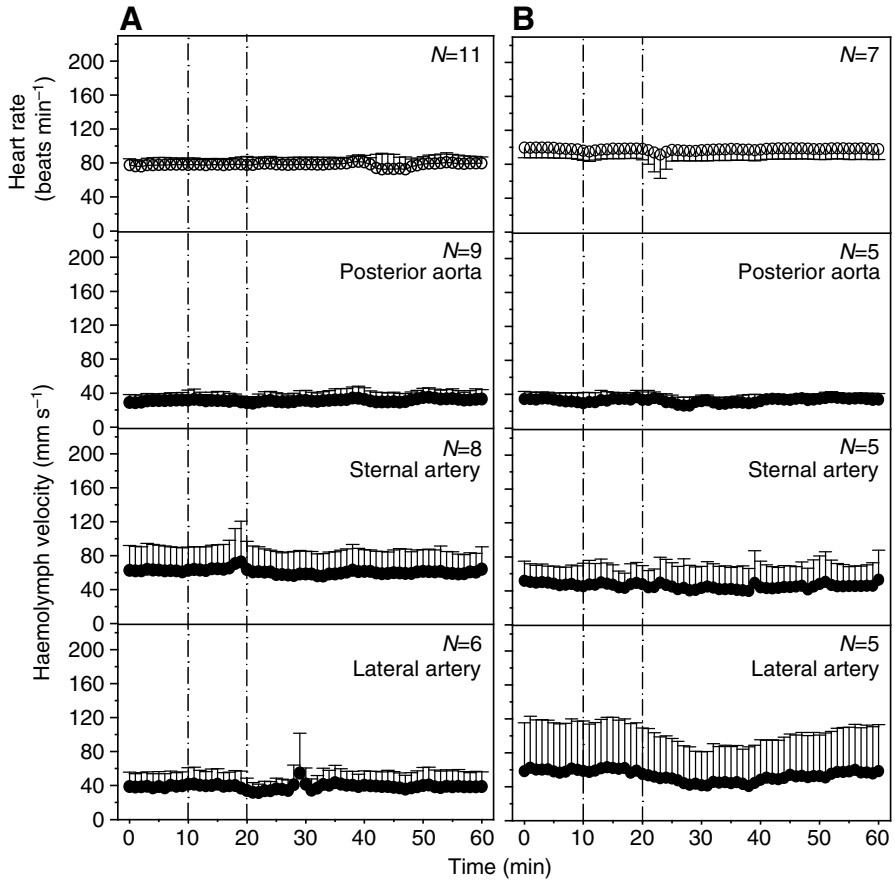


Fig. 5. Comparison of heart rate (beats $min^{-1}$) and haemolymph velocity, $v_{HL}$ (mm $s^{-1}$) in the posterior aorta, the sternal artery and the left lateral artery in intact (A) and cardioregulatory-denervated (B) *Homarus americanus* before ($t_0$–$t_{10}$), during ($t_{11}$–$t_{20}$) and after ($t_{21}$–$t_{60}$) the perfusion of Ringer solution (pH 8.0). The vertical dashed lines indicate the infusion period. Data are presented as means ± s.d. (*N* is given in the respective graphs).

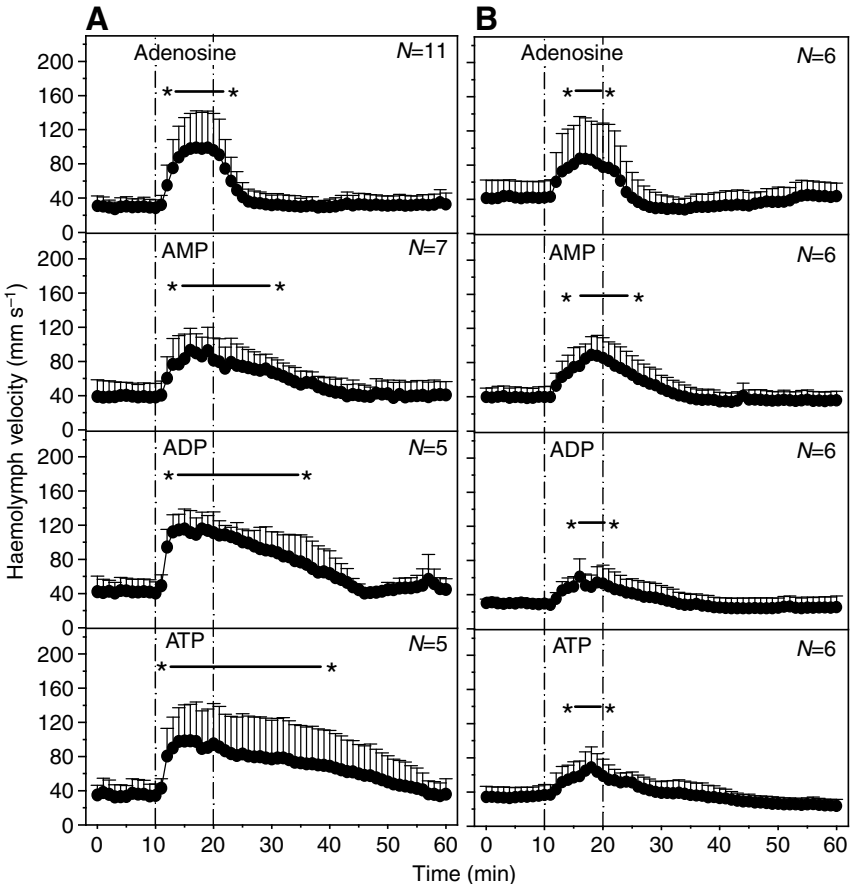


Fig. 6. Comparison of haemolymph velocity, $v_{HL}$ (mm $s^{-1}$) in the posterior aorta in intact (A) and cardioregulatory-denervated (B) *Homarus americanus* before ($t_0$–$t_{10}$), during ($t_{11}$–$t_{20}$) and after ($t_{21}$–$t_{60}$) the perfusion of a solution of 2.4 nmol $min^{-1}$ $g^{-1}$ body mass of adenosine or adenine nucleotides. The vertical dashed lines indicate the infusion period. Data are presented as means ± s.d. (*N* is given in the respective graphs). All values under the lines with asterisks at each end are significantly different from the control value ($P \leqslant 0.05$).

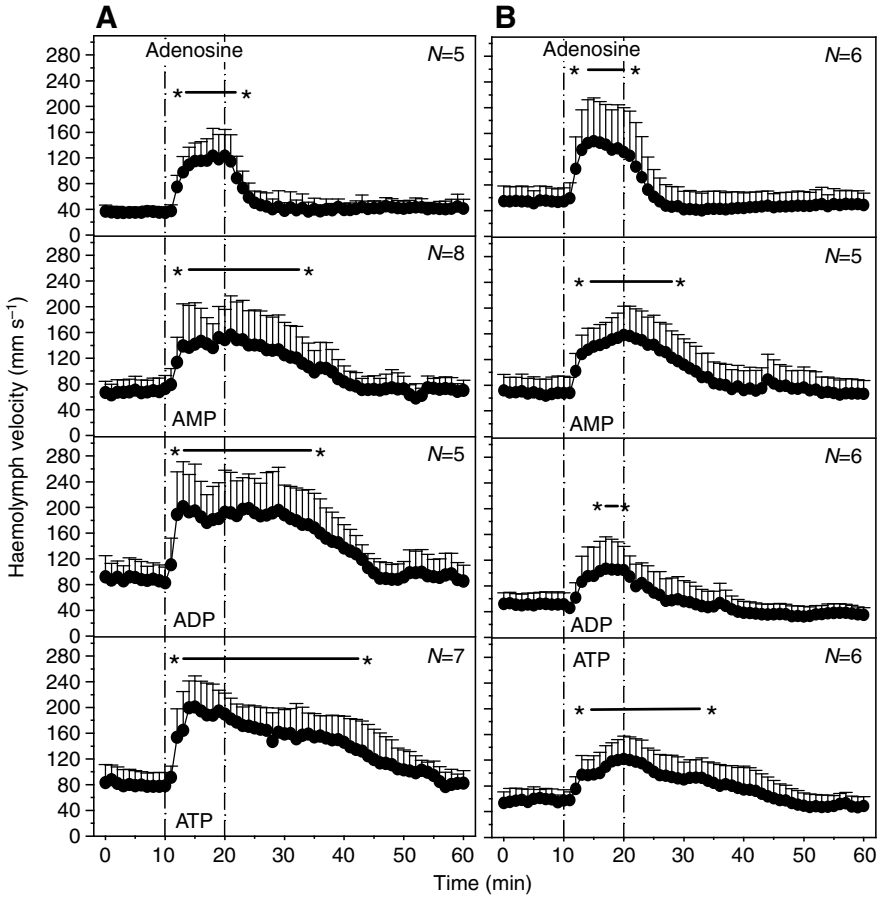


Fig. 7. Comparison of haemolymph velocity, $v_{HL}$ (mm $s^{-1}$) in the sternal artery in intact (A) and cardioregulatory-denervated (B) *Homarus americanus* before ($t_0$–$t_{10}$), during ($t_{11}$–$t_{20}$) and after ($t_{21}$–$t_{60}$) the perfusion of a solution of 2.4 nmol $min^{-1}$ $g^{-1}$ body mass of adenosine or adenine nucleotides. The vertical dashed lines indicate the infusion period. Data are presented as means ± s.d. (*N* is given in the respective graphs). All values under the line with asterisks at each end are significantly different from the control value ($P \leqslant 0.05$).

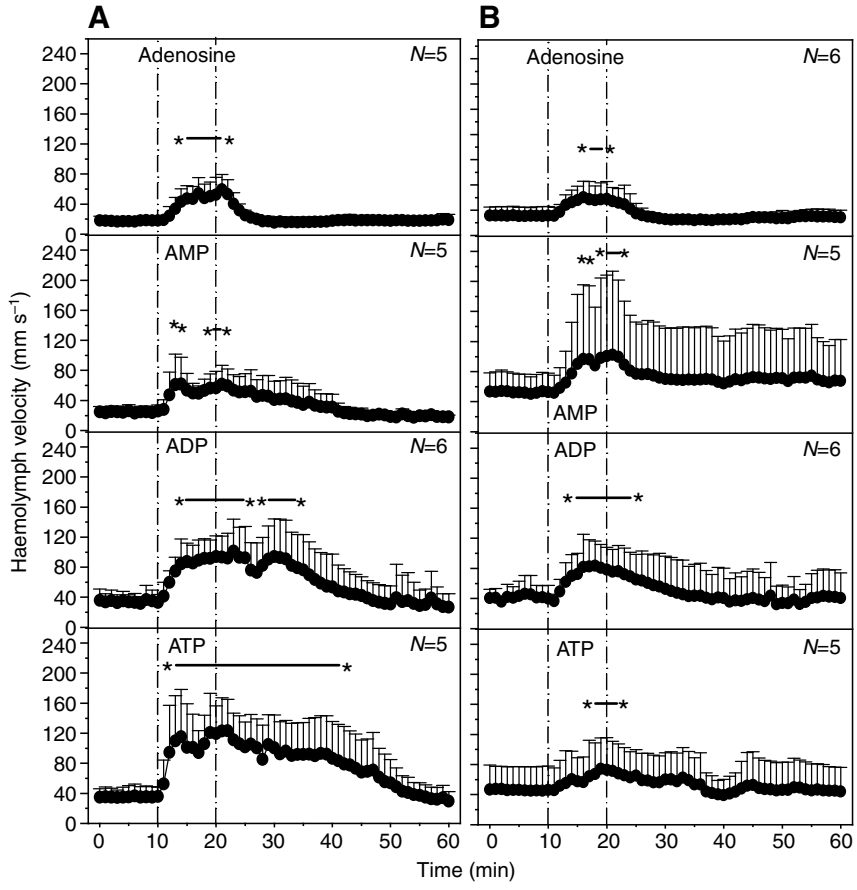


Fig. 8. Comparison of haemolymph velocity, $v_{HL}$ (mm $s^{-1}$) in the left lateral artery in intact (A) and cardioregulatory-denervated (B) *Homarus americanus* before ($t_0$–$t_{10}$), during ($t_{11}$–$t_{20}$) and after ($t_{21}$–$t_{60}$) the perfusion of a solution of 2.4 nmol $min^{-1}$ $g^{-1}$ body mass of adenosine or adenine nucleotides. The vertical dashed lines indicate the infusion period. Data are presented as means ± s.d. (*N* is given in the respective graph). All values under the line with asterisks at each end are significantly different from the control value ($P \leqslant 0.05$).

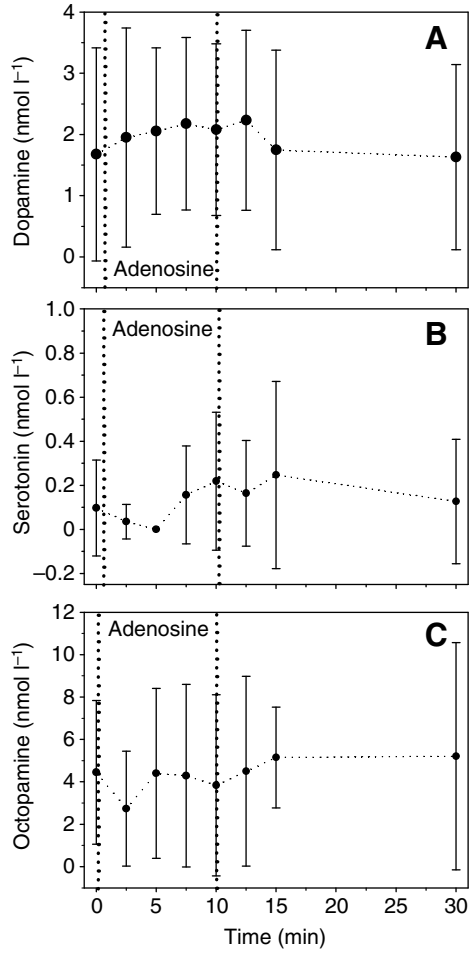


Fig. 9. Haemolymph concentration of dopamine (A), serotonin (B) and octopamine (C) before ($t_0$), during ($t_{2.5}$–$t_{10}$) and after ($t_{12.5}$–$t_{30}$) the perfusion of a solution of 2.4 nmol min$^{-1}$ g$^{-1}$ body mass of adenosine. The vertical dotted lines indicate the infusion period. Data are presented as means ± s.d. ($N$=5).

## DISCUSSION

The cardiovascular organs of decapod crustaceans, e.g. the American lobster, constitute a complex, highly efficient, and tightly regulated system (McMahon and Burnett, 1990; Reiber and McMahon, 1998). To maintain an aerobic metabolism during extreme situations, when oxygen is scarce as during environmental or functional anaerobiosis, systemic modulation by neural, hormonal and metabolic factors allow fine tuning of heart activity, distribution system and respiratory pigments. Adenosine and the adenine nucleotides supplement oxygen transport by increasing $f_H$, $v_{HL}$ and scaphognathite frequency, as shown during the infusion of these metabolites (Stegen and Grieshaber, 2001). The metabolic factors urate and L-lactate increase oxygen affinity of hemocyanin, hence augment oxygen transport (Bridges and Morris, 1986).

In animals, adenosine arises from ATP breakdown in situations when the energy demand exceeds energy supply (Bruns, 1991). Such a situation occurs in the lobster during fight and flight reactions. Forced by a predator, the lobster escapes by flipping its abdomen producing a strong water flow, which pushes the lobster backwards away from the predator. During these vigorous movements a significant increase of the adenosine content was detected in the abdominal muscle of *H. americanus* (E. Stegen, G.M. and M.K.G., unpublished). Following activity, inosine, the first degradation product of adenosine, in haemolymph increased significantly, but adenosine did not. Hence adenosine must have been channeled into the haemolymph, but was deaminated rapidly.

The comparison of the effects of adenosine and adenine nucleotides on different heart preparations should allow inferences on the sites of action. Primarily a differentiation between direct and indirect effects, e.g. those mediated by the CNS and by neurohormones, respectively, should be possible. In addition, the semi-isolated (*in situ*) heart preparation should enable a more specific determination of the sites of action on the heart itself, because chronotropic effects will arise from actions on the rate setting cardiac ganglion, whereas inotropic effects can indicate actions directly on the myocardium.

Adenosine and adenine nucleotides had no effect on $f_H$ of semi-isolated (*in situ*) hearts, thus they do not act on the cardiac ganglion, but they do cause a rapid increase in intact animals, so the drugs must act at some level in the CNS in order to increase activity in the cardioacceleratory nerves. The more gradual and less precipitous chronotropic effects of these drugs in cardioregulatory-denervated animals could arise from gradual neurohormone release from the POs. The CNS connections to the POs remain intact in cardioregulatory-denervated animals and neurohormone release could be stimulated by drug action on the CNS or directly on the POs. Similar conclusions were drawn from observations of $f_H$ in intact and cardioregulatory-denervated lobsters walking on a treadmill (Guirguis and Wilkens, 1995; Rose et al., 1998).

Although such neuromodulatory effects of adenosine have been described for vertebrates (Dobson, 1983; Dobson and Schrader, 1984; Fredholm and Dunwiddie, 1988) and invertebrates [a snail, *Helix aspersa* (Cox and Walker, 1987); mussel, *Mytilus edulis* (Baracco and Stefano, 1990)], in the American lobster adenosine infusion evoked no significant change in the haemolymph concentration of DA, 5-HT and OA. These concentrations were, with the exception of DA, below the physiological threshold which is $10^{-9}$ and $10^{-7}$ mol l$^{-1}$ for DA (Florey and Rathemeyer, 1978), 5 $10^{-9}$ mol l$^{-1}$ for 5-HT (Cooke, 1966) and between $10^{-7}$ and $10^{-8}$ mol l$^{-1}$ for OA (Battelle and Kravitz, 1978). This and the finding by Guirguis and Wilkens (Guirguis and Wilkens, 1995) that DA injection in contrast to 5-HT and OA evoked a more pronounced positive chronotropic effect in cardioregulatory-denervated animals lead to the conclusion that DA is responsible for the slight increase in heart rate during adenosine infusion. The 5-HT and OA levels rather reflect the basic concentrations in the haemolymph rather than mediating a physiological effect *in vivo*. These results demonstrate that adenosine and also the adenine nucleotides play a minor role in the rapid increase in heart rate during exhausting activity by its indirect action on the CNS.

In the semi-isolated (*in situ*) heart preparation $\dot{V}_b$, $P_{vent}$ and $V_S$ were not modulated by adenosine, which implies that adenosine has no direct effect on the semi-isolated heart. By contrast, the adenine nucleotides evoked strong inotropic effects. Whereas ATP and ADP were effective at 2.5 mmol l$^{-1}$, a 5 mmol l$^{-1}$ solution of AMP was needed to increase $P_{vent}$ and $V_S$ significantly (data not shown). The increase of contractile force in the presence of adenine nucleotides indicates a direct effect on the myocardium, which must be mediated *via* purinergic receptors localized on the myocardium. Since the effects are caused by ADP and ATP, but not by adenosine, the existence of P2 receptors (Burnstock, 1996) can be postulated. According to Burnstock (Burnstock, 1996) adenosine and AMP mediate their effects via P1 receptors whereas ADP and ATP mediate their effects by acting on P2 receptors. By contrast,

Illes et al. (Illes et al., 2000) suggested that AMP mediates no effect by its own; instead it has to be degraded to adenosine. These hypotheses, which were proposed for vertebrates may not fit the present observations. Assuming that differences exist between vertebrate and invertebrate receptor types (Hoyle and Greenberg, 1988; Walker et al., 1996), a P2-receptor subtype with low affinity for AMP but with increased affinity in the order ADP<ATP would explain the recorded effects.

The drug-induced increases in $v_{HL}$ in intact and-denervated animals can be partly explained by the inotropic effects produced in semi-isolated hearts. Additionally, the greater velocity in intact animals points to drug actions at some level in the CNS, since denervation reduces this. Hence, these results demonstrate that the change in $v_{HL}$ during exhausting activity was due to an indirect effect of adenosine and the adenine nucleotides. In addition the nucleotides act directly on the heart muscle to increase $v_{HL}$.

Nevertheless, the question still remains of how adenosine or its nucleotides increases $v_{HL}$ in cardioregulatory-denervated animals, although the contribution of the $f_H$ is marginal. At this point only speculation about the effects leading to the increase in $v_{HL}$ is possible.

*In vivo* adjustment of flow occurs by variation of either $f_H$ or $V_S$ or both supplementing each other. Haemolymph velocity (in mm $s^{-1}$) is related to haemolymph flow (in ml $min^{-1}$) by means of the vessel diameter. Assuming that $v_{HL}$ is equal to haemolymph flow, an increase in $V_S$ must be the main origin of the enhanced flow. Owing to the lack of veins, decapod crustaceans do not posses a Frank-Starling-like mechanism (Wilkens and McMahon, 1992) that accounts for an increase in $V_S$ in vertebrates. However, enhancement in $V_S$ in decapod crustacean could occur on different levels.

Enhancement in $V_S$ the contraction properties of suspensory structures, e.g. the elastic ligaments and the pericardial septum. Both structures contain muscle cells and receive innervation (Alexandrowicz, 1932; Maynard, 1960) [Volk, 1988 in McMahon and Burnett (McMahon and Burnett, 1990) in Dungeness Crab, *Cancer magister*]. Modification of the state of contraction could emerge indirectly *via* nerves arising from the CNS or directly *via* purinergic receptors localized on both assisting structures. If so, the adenine nucleotides affect $v_{HL}$ either way, whereas adenosine acts indirectly, as seen in the experiments on the semi-isolated (*in situ*) heart preparation. Nevertheless, to what extent both structures contribute *in vivo* to an increased $V_S$ remains questionable and needs further investigation.

An indirect influence on $V_S$ can also occur through an increase in resistance at the periphery (Wilkens, 1997), as shown by a number of neurotransmitters such as acetylcholine, glutamate, GABA, and neurohormones such as DA, OA, 5-HT, CCAP, PR and F1 and F2 (Davidson et al., 1998; Wilkens and Kuramoto, 1998). Another possibility is through the regulation of the vessel diameter. With the exception of the posterior aorta, arteries in crustaceans lack muscle cells and therefore it is assumed that an active reduction in vessel diameter cannot occur (Martin and Hose, 1995; Wilkens et al., 1997a). However, muscle cells in the posterior aorta respond to PR and electrical stimulation, which *in vivo* might allow active reduction in vessel diameter (Wilkens et al., 1997a; Wilkens et al., 1997b). Additionally, recent studies indicate that an active regulation of the vessel diameter might be possible by stress fibers found in the wall of the lateral arteries (Chan et al., 2006). If such structures were found in all vessels, this point has to be considered.

To increase the resistance actively by adenosine and adenine nucleotides, purinergic receptors must be localized on the valves and/or on the vessels, leading to a contraction of the valves or to vasoconstriction. In addition to a change in $V_S$, modification in vessel resistance might result in a local redistribution of the haemolymph *in vivo*, comparable to the effect of neurohormones acting on the semilunar valves (Kuramoto and Ebara, 1984; Kuramoto et al., 1992; Wilkens, 1997; Wilkens and Kuramoto, 1998). However, an increase in resistance and such redistribution could not be detected in either *in vivo* preparation.

An increase in $V_S$ could also result from an enhancement in stroke power. The increase in myocardial contraction and hence an enhancement in $V_S$ was observed in semi-isolated heart experiments, where PR and F1 increased $V_S$ by direct effect on the myocardium (Saver and Wilkens, 1998; Wilkens, 1999). In this study the perfusion of the semi-isolated hearts with adenine nucleotides produced an increase in ventricular pressure, hence an enhancement in flow, whereas $P_{vent}$ was unaffected by adenosine perfusion. Obviously adenine nucleotides, but not adenosine elicit an inotropic effect by direct action on the myocardium.

Summarizing the results and taking the speculations into account, heart activity in the American lobster, *Homarus americanus*, is modified indirectly by adenosine *via* the CNS and DA, whereas the adenine nucleotides not only modify heart activity indirectly but also directly by action on the heart itself.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| CCAP | crustacean cardio active peptide |
| CNS | central nervous system |
| DA | dopamine |
| $v_{HL}$ | haemolymph velocity |
| $f_H$ | heart rate |
| 5-HT | serotonin |
| OA | octopamine |
| POs | pericardial organs |
| $P_{vent}$ | ventricular pressure |
| $\dot{V}_b$ | cardiac output |
| $V_S$ | stroke volume |

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