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CHOLINERGIC BRADYCARDIA TEMPERATURE INDUCED IN ANTARCTIC FISHES *NOTOTHENIA NEGLECTA* AND *CHAENOCEPHALUS ACERATUS**

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Abstract: The cardiac responses of the Antarctic fishes *Notothenia neglecta* and *Chaenocephalus aceratus* to acute changes of temperature were studied. The fishes were surgically prepared for electrocardiogram (ECG) recording and placed in a three hour experiment in an experimental chamber containing aerated marine water. The temperature was controlled from $+2°C$ to $-1.8°C$. Presence of nitrites in the fish bath was monitored. Atropine and epinephrine were administered into the abdominal cavity through a surgically inserted cannula. Blood samples were taken at initial $+2.0°C$ and final $-1.8°C$ for cholinesterase assay. Results showed that: 1. A temperature decrease from $+2.0°C$ to $\sim -1.8°C$ significantly depressed cardiac rate, from 26.9 ± 2.7 to 5.2 ± 1.4 beats min^{-1} in *N. neglecta* and from 19.0 to 3.0 beats min^{-1} in *C. aceratus*. In one specimen of *N. neglecta*, a reversible cardiac arrest of 3.0 min was observed. ECG waves did not change during the experiments, except the QRS to QRS or the Q-T intervals at lower temperatures. Respiratory rate was not significantly influenced by temperature, 2. Return of temperature to $+1.5/2.0°C$ immediately ceased bradycardia, 3. Administration of atropine reversed bradycardia within 5 min; 4. Serum cholinesterase activity decreased at the lowest temperature; 5. Epinephrine neither influenced the cardiac rate at any temperature nor altered cardiac waves. Results demonstrate that severe bradycardia at subzero temperatures is not only an intrinsic cardiac response to cold, but an active one, due to the higher cholinergic tone, that was not observed in the resting state at $+2.0°C$. No adrenergic influence on heart chronotropism has been found.

key words: Antarctic fish, cholinergic bradycardia, fish cardiac arrest, *Notothenia neglecta*, *Chaenocephalus aceratus*

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Introduction

Antarctic fishes are a unique model of survival at temperatures close to the freezing point of sea water, a thermal condition that would be lethal to any other fish (for further reviews on the biology of Antarctic fish see MacDonald *et al.*, 1987; Di Prisco *et al.*, 1991; Eastman, 1993). Enzymatic adaptation to such a rough environment suggests a high level of specialization (Somero, 1991). Doubt arises about the thermal optima and thermal limit for enzymatic function that would allow appropriate biochemical and physiological performance. Acetylcholinesterase (AChE) activity is critical for cholinergic synaptic transmission, acting in a number of vital neuroeffector synapses, therefore playing an important role whenever acetylcholine (ACh) acts as a chemical neurotransmitter. Enzyme thermal disturbance might underlie some neurological impairment in Antarctic fishes, due to uncoupling of physiological control of "transmitter release-transmitter hydrolysis" (Somero and DeVries, 1967). A sharp effect of temperature has been described on the Km of acetylcholine for acetylcholinesterase in the Antarctic fish *Pagothenia borchgrevinki* (Baldwin and Hochachka, 1970; Baldwin, 1971). Important neurohumoral modulation is implicated in both acute and long-term adaptations to temperature of the teleosts heart (Laurent *et al.*, 1983; Farrel, 1984). The heart of freshwater fishes is, as a rule, innervated by the vagus nerve (Mott, 1957; Randall, 1966, 1968; Bone *et al.*, 1995). The stimulation of this nerve causes bradycardia which can be blocked by atropine while application of acetylcholine slows the heart (Johansen *et al.*, 1966). Adrenergic fibers have been found to innervate the heart of teleost fishes (Gannon and Burnstock, 1969). Occurrence and availability of α and β-adrenoceptors are implicated in adaptations to temperature of teleosts heart (Laurent *et al.*, 1983; Farrel, 1984) while circulating catecholamine influences the heart rate (Bone *et al.*, 1995). Nervous and humoral heart control in Antarctic fishes is less known. Cholinergic and adrenergic innervation has been demonstrated in fishes. Axelsson *et al.* (1992) noted that in *Pagothenia bernachii* and *P. borchgrevinski*, a resting heart rate is the result of an inhibitory cholinergic tone of 80.4% and an excitatory adrenergic tone of 27.5%. Furthermore, the intrinsic heart rate was 21.7 beats min^{-1} at 0°C. Very important also in regard to this research is the observation by Hoshino *et al.* (1987) of reversible cardiac arrest induced by startling stimuli in *Notothenia neglecta.* Long cardiac arrests in this fish were also observed by Bastos-Ramos *et al.* (1995).

In this paper we intended to study the cholinergic role in bradycardia induced by decreasing the temperature in the Antarctic fish *Nothothenia neglecta*, and responses to temperature in the ice fish *Chaenocephalus aceratus*. Preliminary results of this work were presented at the XIX Symposium on Polar Biology, Tokyo (Bastos-Ramos *et al.*, 1996a).

Material and Methods

Specimens of *Notothenia neglecta* and of the ice fish *Chaenocephalus aceratus* were caught in the Admiralty Bay, South Shetlands, Antarctica, at the Brazilian Antarctic Station during the Antarctic Summer of 1994–1995. The fishes were caught by a special three-mesh bottom net at 80–100 m depth. *N. neglecta* was the easiest species to catch

while *C. aceratus* were difficult to catch in good condition because they did not survive or were often caught dead, devoured by amphipod. From 6 to 10 specimens of *N. neglecta* were kept in 1000 *l* indoor tanks for 7 days acclimation, at temperatures of +0.5 to 1°C. The water was monitored in regard to pH and salinity and renewed once or twice a day according to the aquarium population, maintained at a maximum of 10 fishes. The aquarium was cleaned daily of feces and mucus. The room was dimly lighted, noise being avoided as much as possible. A total of 39 *N. neglecta* and 3 *C. aceratus* specimens of 30–40 cm length and in good health were used for the experiments.

Prior to surgical procedures, the fishes were anesthetized with benzocaine (1 : 10000). Surgery was performed in a cold room with the air temperature at a maximum of 5°C. The anesthetic solution was maintained at 0–1°C, bubbled with air. In order to perform the electrocardiogram (ECG) recording, the anesthetized animals were placed on an operating table and prepared as follows. Two electrodes made of stainless steel hypodermic needles, connected to flexible isolated cables, were surgically implanted into small incisions made in the skin and muscle. One of them was inserted into the pericardial area and the other into the muscle of the abdominal region, both in the midline of the ventral surface. The electrodes were fixed by surgical sutures and isolated with cyanoacrylate glue. The skin incision was sutured and then covered with the glue to assure perfect isolation. After insertion of the electrodes, most of the fishes were put back in the aquarium and the experiments performed some days later. For the administration of drugs, a polyethylene tube was inserted through a 1.5 mm incision into the abdominal cavity. The cannula was fixed by a surgical suture and the wound sutured and covered with cyanoacrilate glue. Blood samples were obtained from the caudal vein by means of a vacuum tubing system.

To do the experiment the fishes were placed in a Plexiglas double walled experimental chamber as described by LUCCHIARI *et al.* (1984) The internal chamber was filled with 5.2 *l* of sea water, bubbled through an air stone with precooled air. The external compartment was filled with circulating cooling solution (ethylene glycol 30%) supplied by a two way thermostatic circulating pump, which could control the marine water temperature of the inner chamber. The electrode cables were connected to the electrocardiograph recorder. The experimental chamber was then enclosed in an isolating cover, in order to avoid light and sound. A small window could be opened to monitor the fishes' respiration. The electrical system was grounded.

Before the beginning of the experiments the fishes were allowed to habituate in the chamber for about 30 min and a baseline ECG was recorded. Habituation was indicated by a steady heart rate. The temperature was gradually decreased from +2.0°C to −1.8°C in a 3-hour experiment. Blood samples were collected at the two extreme temperatures. Atropine, 25 mg kg^{-1} (6 experiments) or epinephrine, 50 $\mu g\, kg^{-1}$ (4 experiments) was administered at the lower temperature. In 12 experiments the temperature was raised from −1.8 to +1.5/2.0°C. The rate of temperature increase was approximately of 0.2°C min^{-1} and was achieved by gradual change of the chamber water. As a control, in 5 experiments the temperature of the chamber water was kept the same for 3.0 hours and the ECG recorded.

The sea water salinity and the pH were monitored using an Ingold-pH-206 monitor. During the experiments, nitrites in the chamber were assayed according to the method

of EGAMI and TANIGUCHI (1963).

To obtain serum, whole blood was spun down at 440 *g* for 30 min at around 0°C; the supernatant serum was collected and used for cholinesterase activity assay and for protein determination. For cholinesterase studies, blood was collected by venous puncture of the caudal vein, from *N. neglecta* specimens acclimatized at +2°C and at −1.8°C. Immediately upon centrifugation, serum cholinesterase was assayed at 37°C according to the technique of DIETZ *et al.* (1973). Serum total protein was assayed according to LOWRY *et al.* (1961).

Statistical analysis was carried out by Student's "*t*" test and multivariate variance analysis.

Results

When the water temperature in the fish chamber was decreased from +2.0°C to −1.8°C severe bradycardia, from 27 ± 3.1 to 5.6 ± 2.5 beats min^{-1} in *N. neglecta* and 19 to 3 beats min^{-1} in *C. aceratus* was observed. Such bradycardia could last up to 7 hours and the fish survived. Increasing the temperature up to +2.0°C immediately reversed the bradycardia. In *N. neglecta* it returned to 23 ± 3.2 beats min^{-1} and in *C. aceratus* to 14.0 beats min^{-1}. In 9 fishes the cardiac rate was as low as 3 beats min^{-1} and in one, reversible cardiac arrest that lasted 3 min was observed (Figs. 1 and 2). After the end of the 3-hour experiment, the ECG continued to be recorded up to 6–7 hours. This step was used as a control for the whole experiment. Furthermore, only moderate bradycardia has been observed (see Fig. 1). No modifications of P, T or QRS but increased intevals between consecutive QRS and between Q and T were observed. Light but not significant respiratory depression was observed at subzero temperatures.

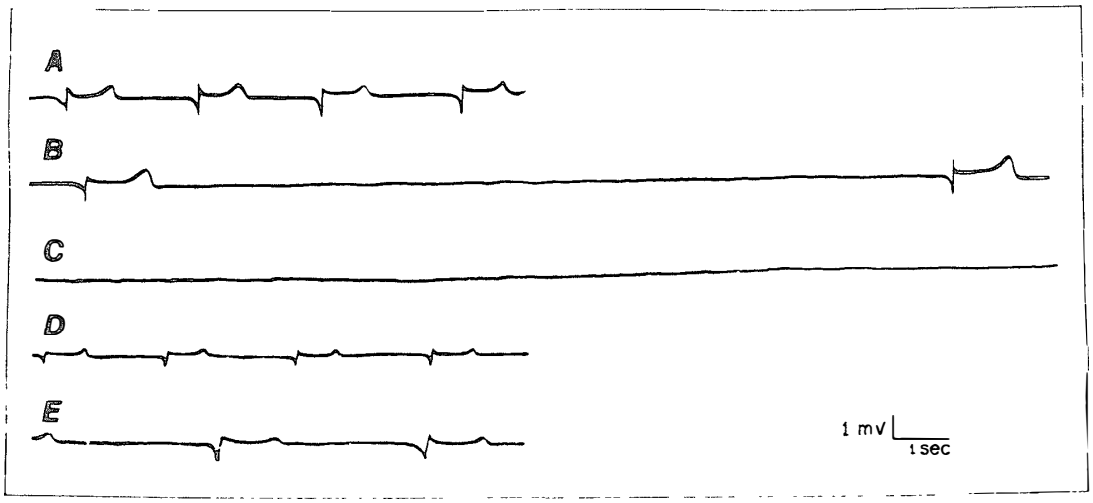

Fig 1 Electrocardiogram of *Notothenia neglecta* performed in lead DI. Derivation DI has been used, corresponding to the nomenclature used for ECG in man. right arm and left arm A Initial, at +2 0°C, cardiac rate = 23 beats min^{-1}, B At −1.8°C, cardiac rate = 3 beats min^{-1}, C. Reversible cardiac arrest lasting 3 min, D Return of temperature to +1 5°C, cardiac rate = 24 beats min^{-1}, E ECG after 6 hours at 0°C, cardiac rate = 14 beats min^{-1}

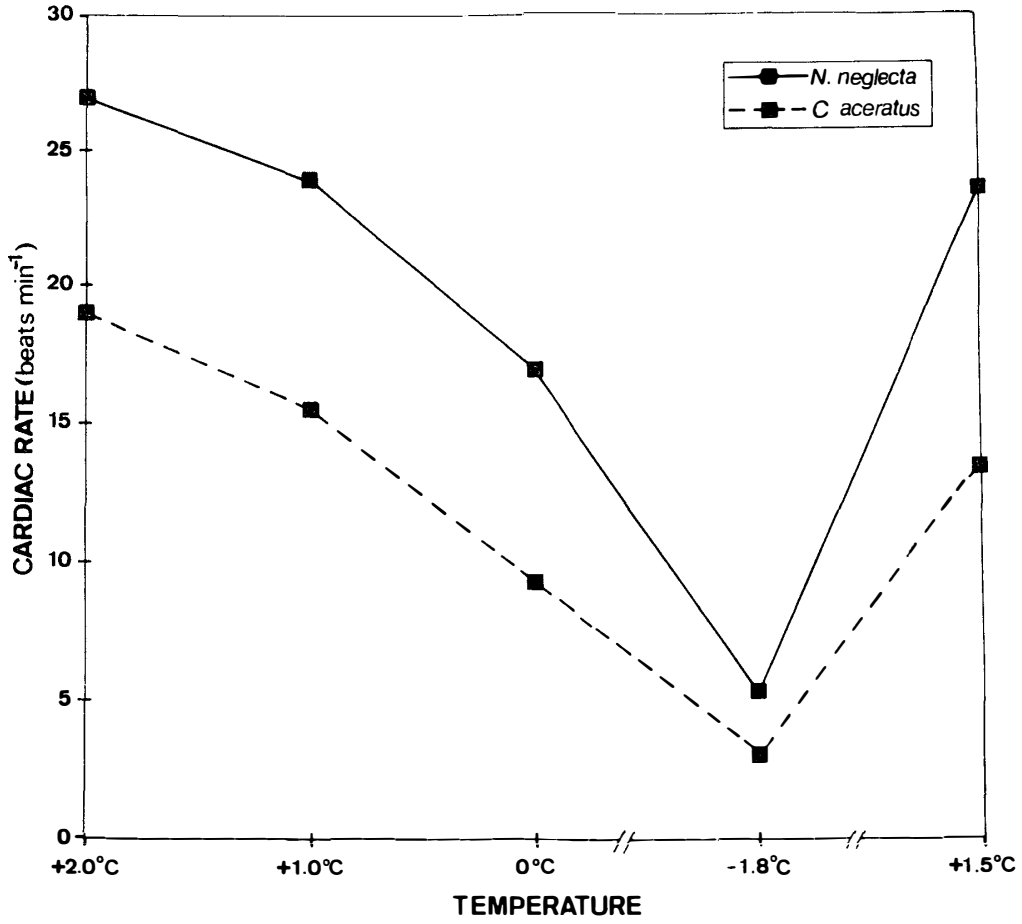

Fig. 2 Influence of the temperature on the cardiac rates, in beats min^{-1}, displayed by *Notothenia neglecta* and *Chaenocephalus aceratus*. Ranges of temperature used: from +2.0°C to −1.8°C and return to +1.5°C. Time of the experiment: 3 hours. Heart beats in min^{-1} for *N. neglecta* (n=22): 26.9±2.69, at +2.0°C; 23.9±4.4, at +1.0°C; 17.0±2.0, at 0°C, 5.3±2.0, at −1.8°C; 23.6 ± 3 2, at +1.5°C. Mean values of beats min^{-1} for *C aceratus* (n=2): 19.0 at +2.0°C; 15.5 at +1.0°C, 9.3, at 0°C; 3.0, at −1.8°C, 13.5, at +1.5°C

At temperatures of about +1.5°C and above, heart beats and respiration occur in a similar rhythm. However, such similarity is not observed at lower temperatures (Fig. 4).

In five specimens of *N. neglecta*, maintained at −1.8°C, atropine administered at the abdominal cavity gradually reverted bradycardia. The reversion started 5 min after administration of the drug and reaching maximum values comparable to the initial ones (Fig. 3). Administration of intra-abdominal epinephrine during bradycardia failed to alter the cardiac rate or ECG waves. The drug caused a whitish body response in the fish, starting 5 min after its administration, an effect that lasted about 60–90 min. An excitatory state was observed and sometimes death of the animal was caused by this effect. Note that −1.8°C is very close to the sea water freezing point. The fish always died when, occasionally, ice started to form in the water bath in which the experiment was being carried out.

Serum cholinesterase activity was significantly influenced by the temperature to which the fish were acclimatized. There was a decrease in specific activity values for

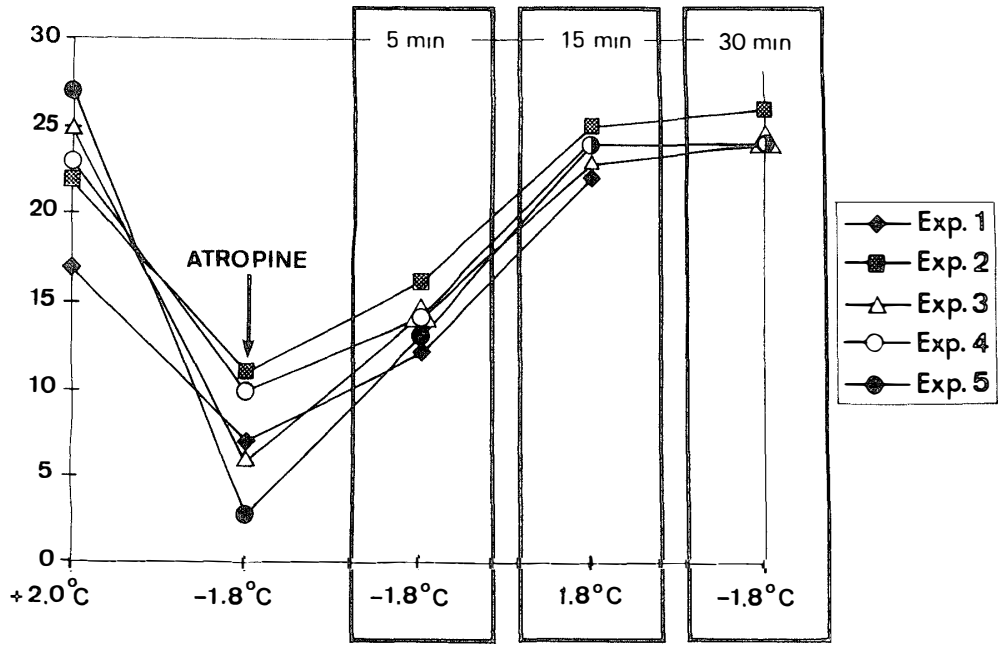

Fig. 3. The effect of atropine on the cardiac rate of *Notothenia neglecta* Values in beats min^{-1} were established at +2.0°C and at −1.8°C before and 5, 15 and 30 min after the administration of atropine (25 mg kg^{-1}) in the abdominal cavity The figure shows individual values (n = 5). The inflection point corresponds to the administration of atropine.

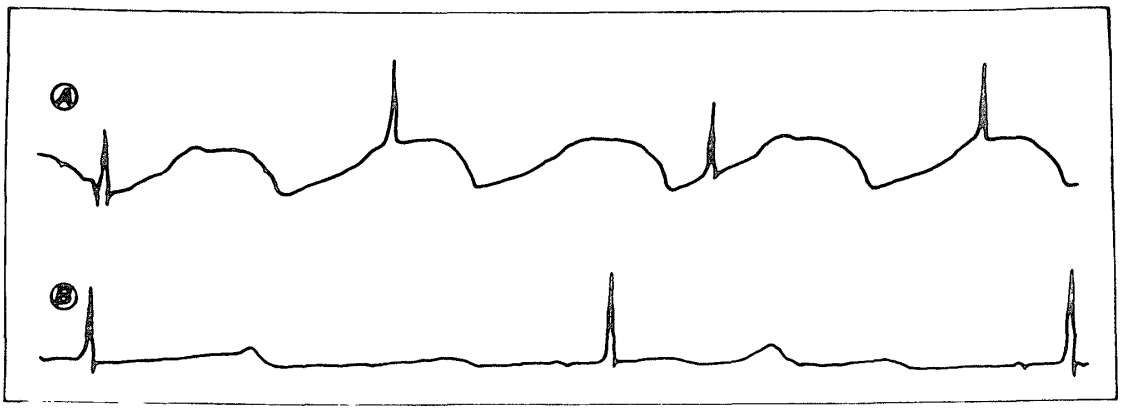

Fig. 4. Heart beats and respiratory movements displayed by the electrocardiogram of *Notothenia neglecta* Coordination between heart beats and respiratory movements is shown as sinusoidal curves when recorded at +1 5°C. A. At +1.5°C, cardiac rate = 30 beats min^{-1}, respiratory rate = 32 min^{-1} B. At 0°C, cardiac rate = 18 beats min^{-1}, respiratory rate = 31 min^{-1} At temperatures lower than 1 0°C there is no correlation between heart beats and respiratory movements

the enzyme assayed in blood collected from fish acclimatized at 2.0°C compared to samples collected from fish acclimatized at −1.8°C (Table 1).

The control fishes were maintained at a constant temperature (1.5–2.0°C) for up to 6–7 hours. With these animals, moderate bradycardia of 5–40% of initial values was

Table 1 Serum cholinesterase, expressed as specific activity, in *Notothenia neglecta* acclimatized at +2.0°C and at −1.8°C. Blood samples were collected from the caudal vein, spun down at about 0°C and serum cholinesterase immediately assayed at 37°C. *Cholinesterase specific* activity was calculated as the ratio between units of enzyme per *l*/g protein per *l*.

No.	Temperature		Decrease in activity (%)
	+2.0°C	−1.8°C	
1	0.275	0.163	40.8
2	0.225	0.169	24.9
3	0.225	0.168	25.4
4	0.255	0.183	28.3
5	0.269	0.162	39.8
6	0.260	0.174	33.1
Mean	0.241	0.170	
SD*	0.002	0.001	

* SD = Standard deviation

observed.

Salinity of sea water ranged from 32 to 34‰ and the pH was 7.22 ± 0.25. No nitrite residues were found in the experimental chamber during the three hour experiments.

Discussion

Results indicate that temperature is very important to establish physiological and biochemical parameters in Antarctic fishes. In this paper a study was carried out on the cardiac and respiratory responses of the fish in acute experiments in which the temperature was decreased from +2.0°C to −1.8°C in a 3 hour interval. A shorter experimental time was not used because it could cause stress, while a 3 hour gradual change of temperature would allow some habituation of the animal. In fact, no stress response, such as restless behavior, the presence of mucus or change in color to a whitish body, was observed. Benzocaine induced anesthesia within 7–8 min, lasting about 10–14 min, just enough for the surgical procedures. In the doses used, no respiratory depression was observed, so that it can be considered quite suitable for the anesthesia of Antarctic fishes. Some animals were put back in the aquarium right after the surgery, while others were immediately used for the planned experiments. No difference in the results or stress signs was observed among them.

The experimental model proposed in this paper, in which the fishes are submitted to programmed temperatures for a short time, seems to be suitable for comparative studies on the influence of temperature upon physiological and biochemical parameters. The adequacy of the experimental system was demonstrated by (a) lack, as a rule, of catabolic products in the water of the fish chamber for 3 hours; (b) in the control animals, ECG, as a parameter of physiological condition, did not alter during the 3 hours experiments. It is then unnecessary to use a fish chamber with running water, which could induce serious technical difficulties in achieving sharp control of different

temperatures. The bradycardia observed in long lasting observations (6–7 hours), cannot be attributed to hypoxia, as pointed out by NAMBA *et al.* (1987), RANDALL (1970) and GLASS *et al.* (1991), in experiments with trout, since the bath water was always saturated with bubbling air. On the other hand, no cardiac arrhythmia was observed.

Slowing of the heart rate at lower temperatures is quite predictable and a well known phenomenon that probably represents an intrinsic compensatory mechanism by which the heart finds a balance to lower metabolic needs. In our experiments bradycardia as low as 3 beats min^{-1} was observed. In one specimen of *N. neglecta*, a reversible cardiac arrest of 3 min was observed, One may suppose that higher cardiac output during bradycardia maintained the tissue perfusion. As described with trout by RANDALL (1970), bradycardia that developed during hypoxia is compensated by an increase in stroke volume in such a way that only minor changes in cardiac output could be observed In our experiments, drugs (atropine and epinephrine) administered during severe bradycardia were absorbed and distributed as a result of circulatory efficiency.

Coordination of cardiac beats with respiratory movements, with a ratio nearly 1 : 1, was observed when the fishes were maintained at $+2.0°C$ (Fig. 4). With decrease of temperature, the cardiac rate progressively diminished but respiratory movements were not significantly affected. Cardiorespiratory coordination has been observed in rainbow trout *Oncorhyncus mykiss* (RANDALL and SMITH, 1967) and in carp *Cyprinus carpio* L. (GLASS *et al.*, 1991).

Administration of autonomic drugs like atropine and epinephrine through an intra-abdominal cannula was found to be very appropriate for fish. Use of a previously inserted cannula, besides being easily performed and less invasive, avoided the stress that normally occurs by injecting those compounds directly by means of intravascular cannulation. A good rate of absorption of these drugs through the abdominal mucosa was demonstrated by the responses to both compounds. Starting 5 min after the administration, atropine causes cardiac reversal of bradycardia while epinephrine induces whitening of the fish body. A delay in the physiological responses could be due either to slower absorption as compared to an intravascular route and/or to slower reaction at specific receptors. On the other hand, high doses of the drugs (25 mg kg^{-1} of atropine and 50 $\mu g\,kg^{-1}$ of epinephrine) chosen after initial trials, were necessary to achieve clear results. AXELSSON *et al.* (1992), in experiments with the Antarctic fish *Pagothenia bernachii* and *P borchgrevink*, used smaller doses of atropine (1.2 mg kg^{-1}) and epinephrine (10 nmol kg^{-1}) injected through a cannulated aorta. In parallel experiments similar results were obtained with *N. neglecta* by means of an intravenous route. Absorption through the abdominal mucosa is gradual and less aggressive. Epinephrine is poorly absorbed from the abdominal mucosa in animals but it could cross through biological membranes to enter the circulation in Antarctic fishes.

Cardiac cholinergic and adrenergic tones are known to be important in cardiac control of fishes (MOTT, 1957; JOHANSEN *et al.*, 1966; RANDALL, 1966, 1968, 1970; YAMAUCHI and BURNSTOCK, 1968; GANNON and BURNSTOCK, 1969; AXELSSON *et al.*, 1992; BONE *et al.*, 1995). In our experiments with *N. neglecta* cholinergic tone was shown to play an important role in bradycardia at subzero temperatures since it was blocked by atropine. The decrease in the rate of serum cholinesterase activity at $-1.8°C$ (Table 1) seems to be in agreement with this proposition. This may account for the fact that

more acetylcholine might remain unhydrolyzed acting upon cholinergic receptors. This will enhance parasympathetic heart responses. Since the specific activity of serum cholinesterase is much lower at $-1.8°C$ than at $+2.0°C$, it is possible that the rate of protein synthesis might also be influenced by the lower temperature to which the fish is biologically adapted. Considering that the values of specific activity of serum cholinesterase from *N. neglecta* adapted to $-1.8°C$ are lower than the ones found in specimens adapted to $+2.0°C$, it may be possible to conclude that this might be a consequence of the lower rates of protein synthesis. It may also be that at subzero temperatures impairment of the binding of acetylcholine to the active site of the enzyme might occur. On the other hand, one can suppose that the enzyme-substrate complex formation is slower at $-1.8°C$ and might also be slower in the Antarctic fish *N. neglecta* than in freshwater fishes adapted to higher temperatures. According to BASTOS-RAMOS *et al.* (1996a, b) the organophosphorous agent Malathion inhibits cholinesterase from the Antarctic fish *N. neglecta* in a much slower pattern than that found for Parathion in the tropical fish *Challichthys challichthys* by SILVA *et al.* (1993).

Differently from what has been described by AXELSSON *et al.* (1992) for *P. bernachii* and *P. borchgrevinki*, resting heart rates of *N. neglecta*, adapted to $+2.0°C$, were not influenced by such an important cholinergic tone. This can be demonstrated by the fact that atropine did not influence the cardiac rate at that temperature.

Adrenergic tone could not be demonstrated in *N. neglecta* because epinephrine failed to increase cardiac rate, either at $+2.0°C$ or during bradycardia at $-1.8°C$. However, in freshwater fishes, adrenergic tone is probably increased during exercise presumably through adrenergic nerves, because circulating cathecolamine does not increase appreciably during sustained swimming (BUTLER, 1986). Further experiments are advisable to clear up this point.

In studies of mechanical performance of the hemoglobinless icefish *Chionodraco hamatus* isolated and perfused heart, TOTA *et al.* (1991) showed that the increase of temperature from 0.6 to 5.8°C caused a significant increase in heart rate, cardiac output and power cardiac output. They suggested that such responses were basically independent of extrinsic nervous control. It has also been pointed out that nothing was known of the innervation and occurrence of adrenergic receptors in the icefish heart. Preliminary experiments showed (Fig. 2) that the heart rate of the icefish *Chaenocephalus aceratus* was 19 beats min^{-1} at $+2.0°C$, a value slightly higher than those found by HOLETON (1970) and by HEMMINGSEN and DOUGLAS (1977). Cardiac rate decreased proportionally to the lowering of temperature, down to 3 beats min^{-1}. The rising of the temperature to $+1.5°C$ immediately reversed bradycardia as in the red blooded *N. neglecta* (Fig. 2). This bradycardia is probably due to an increased cholinergic tone. However, no data are yet available to clearly explain the possible implication of nervous control of the bradycardia as described for *C. aceratus* at lower temperatures.

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