

## AN EXAMINATION OF CENTRAL CHEMOSENSITIVITY IN AN AIR-BREATHING FISH (*AMIA CALVA*)

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### Summary

The role of central chemosensitivity in the control of ventilation in fishes was investigated directly by perfusing a mock extradural fluid (EDF) through the cranial space in the medullary region of conscious air-breathing fish, *Amia calva*. Perfusions with Sudan Black dye showed that the mock EDF communicated with the cerebrospinal fluid (CSF) and entered the cerebral ventricles. Altering the  $P_{O_2}$ ,  $P_{CO_2}$  and/or pH of the mock EDF had no effect on gill- or air-breathing rates, heart rate or blood pressure during exposure to normoxic water. Aquatic hypoxia, however, stimulated gill ventilation and elevated blood pressure, but did not affect heart rate; altering the gas tensions and/or pH of mock EDF still had no effect on recorded variables. Sodium cyanide (NaCN) added to the mock EDF caused struggling at concentrations above  $500 \mu\text{g ml}^{-1}$ , but did not uniformly stimulate ventilation. These results suggest that central chemoreceptors, which mediate cardiovascular or ventilatory reflexes, are absent in *Amia*.

### Introduction

Nearly all fish studied to date respond to aquatic hypoxia with increased ventilation, but the location of the receptors mediating this reflex is unknown (see Jones, 1983; Shelton *et al.* 1986, for reviews). Previous experiments that have investigated chemoreceptive sites controlling ventilation in fish have yielded equivocal results. For instance, ventilatory hypoxic reflexes persist after branchial nerve section of cranial nerves IX and X in tench (*Tinca tinca*, Hughes and Shelton, 1962) and sea raven (*Hemipterus americanus*, Saunders and Sutterlin, 1971). These observations have led some authors to speculate that central nervous system chemoreceptors in fish may control the ventilatory responses to hypoxia, since peripheral sites cannot be clearly delineated (Bamford, 1974; Jones, 1983). More recent experiments using channel catfish (*Ictalurus punctatus*), however, suggest that peripheral sites are solely responsible for hypoxic chemoreception in this species since ventilatory reflexes are abolished following complete branchial denervation (Burleson and Smatresk, 1986).

Key words: *Amia calva*, fish, hypoxia, hypercapnia, chemoreceptors, ventilation.

Intracranial chemoreceptors sensitive to the  $P_{\text{CO}_2}$  and/or pH of the cerebrospinal fluid (CSF) are important in ventilatory control in mammals (O'Regan and Majcherczyk, 1982). Although there is no clearly established anatomical location for these receptors, they appear to be localised in the ventrolateral medulla oblongata (Mitchell *et al.* 1963; Shams, 1985). Central chemoreceptors responding to changes in  $\text{CO}_2$  and/or pH resulting in increased ventilation have also been demonstrated in the turtle (*Pseudemys scripta elegans*, Hitzig and Jackson, 1978) and toad (*Bufo marinus*, Smatresk and Smits, 1989), implicating an early evolutionary origin for central nervous system  $\text{CO}_2$ /pH chemosensitivity in ventilatory control.

There are few published investigations of potential central chemosensitivity in fish. Hughes and Shelton (1962) reported that microliter injections of sodium bicarbonate/carbon dioxide solutions into the medulla of tench resulted in both transient increases and decreases of ventilation. Rovainen (1977), using an isolated lamprey brain preparation, reported that bathing the brain with  $\text{N}_2$ -equilibrated fluid had no effect on the discharge frequency recorded from central roots of the cranial nerves IX or X; HCl and cyanide added to the bathing medium, however, usually increased the discharge frequency. These studies suggest that some central chemosensitivity to low-pH solutions and  $\text{O}_2$  chemoreceptor stimulants may exist in fish.

*Amia calva* is a primitive air-breathing fish with a bimodal breathing pattern. This species normally uses its gills to extract  $\text{O}_2$  and excrete  $\text{CO}_2$  in water, but supplements  $\text{O}_2$  uptake by gulping air and storing it in the swimbladder (Johansen *et al.* 1970). There is also evidence that aquatic hypercapnia, at concentrations up to 3%  $\text{CO}_2$ , increases branchial ventilation (Johansen, 1970). Such extant air-breathing fish, therefore, possess physiological adaptations that may reflect changes associated with the transition from aquatic to aerial environments. Since *Amia* show aerial and branchial ventilatory responses to both aquatic hypoxia and hypercapnia, studies of central chemosensitivity in these fish not only address the question of whether fish possess central chemosensitivity to  $\text{O}_2$ , but also explore the phylogenetic origins of central  $\text{CO}_2$  or pH sensitivity as demonstrated in terrestrial vertebrates. In this study, we have examined the potential role of central chemoreception to both  $\text{O}_2$  and  $\text{CO}_2$ /pH in the control of breathing in conscious *Amia* by experimentally manipulating the composition of the extradural fluid surrounding the brain.

## Materials and methods

### *Animals*

*Amia* were obtained from a commercial supplier in Ontario and air-freighted to the University of British Columbia. Fish were in good health upon arrival and were maintained in large circular tanks in dechlorinated tap water at 8–15°C on a natural photoperiod. *Amia* were fed live goldfish once per week.

*Surgical preparation*

Fish were initially anesthetized in a 1:10 000 buffered solution of tricaine methanesulfonate (MS 222), and then transferred to a surgical table and artificially ventilated with a dilute amount of oxygenated anesthetic (1:20 000). The dorsal aorta was cannulated with PE 50 using a canine catheter placement unit, as described by Smatresk and Cameron (1982). A cannula (PE 160) to monitor ventilation was implanted in the buccal cavity *via* a hole drilled through the nasal bone.

Each fish was prepared for cranial perfusion by drilling two small holes in the mid-sagittal plane of the cranium using a dental drill. A posterior hole was made first. The hole was drilled at an angle 10–15° from perpendicular, and 2–5 mm from the posterior margin of the parietal bone. The second hole was drilled, at approximately the same angle as the first hole, in the frontal bone about 10 mm anterior to the first hole. Fluid samples were taken at this time with a syringe and placed into capillary tubes for later ion analysis. The fluid was extradural fluid (EDF), and not true CSF, since it was sampled from the meningeal space (see Davson, 1967). Blood was present in some EDF samples and was subsequently removed by centrifugation. Perfusion cannulae made from the shanks of 18 gauge stainless-steel hypodermic needles, approximately 15–20 mm long, were implanted in the cranial holes. The needle shafts were forced through holes in a 7 mm×13 mm aluminum plate and fixed by epoxy cement. The aluminum plate with the implanted cannulae was attached to the cranium with four small stainless-steel screws. The steel tubing extended down into the meningeal space about 10 mm in the posterior (inflow) port and 6 mm in the anterior (outflow) port. It was found in preliminary experiments that this configuration situated the inflow tube about 2 mm above the roof of the fourth ventricle and the outflow tube above the cerebellum. Patency of the perfusion arrangement was then checked by perfusing mock EDF with a syringe connected to the inflow tube. Animals in which flow could not be freely maintained, or in which there was excessive bleeding, were not used in the experiment. Once patency was ensured, the perfusion tubing and meningeal space were filled with mock EDF and plugged. The fish were subsequently transferred to a darkened Perspex box with continuously flowing, normoxic water. The box had a forward air space to allow air-breathing. Surgery seldom required longer than 20 min to complete and all fish recovered quickly after withdrawal of the anesthetic.

EDF concentrations of Na<sup>+</sup> and K<sup>+</sup> were determined by flame photometry (IL model 143); Cl<sup>-</sup> concentration was determined by coulometric–amperometric titration of chloride ions using a Buchler digital chloridometer.

*Protocol*

Experiments began after a 24 h recovery period and were conducted at water temperatures between 14 and 16°C. The temperature throughout a single experiment did not vary by more than 0.5°C. Mock EDF was freshly made with

the following composition: NaCl (120 mmol l<sup>-1</sup>), KCl (4 mmol l<sup>-1</sup>), MgSO<sub>4</sub> (1 mmol l<sup>-1</sup>), CaCl<sub>2</sub> (1 mmol l<sup>-1</sup>) and NaHCO<sub>3</sub> (10 mmol l<sup>-1</sup>). This fluid was placed into 500 ml bell jars which were maintained at the same temperature as the fish throughout the experiment. The EDF was pre-equilibrated with one of four gas mixtures: (1) air ( $P_{O_2}=20.8\pm 0.13$  kPa, pH=7.77±0.02); (2) 100% N<sub>2</sub> ( $P_{O_2}=0.6\pm 0.09$  kPa, pH=8.17±0.03); (3) 100% O<sub>2</sub> ( $P_{O_2}=93.8\pm 1.12$  kPa, pH=8.03±0.04); (4) 3% CO<sub>2</sub> in air [three fish;  $P_{O_2}=19.3\pm 0.5$  kPa;  $P_{CO_2}=2.8$  kPa (calculated); pH=7.02±0.03] or 5% CO<sub>2</sub> in air mixture [five fish;  $P_{O_2}=20.1\pm 0.33$  kPa,  $P_{CO_2}=4.7$  kPa (calculated), pH=6.74±0.01]. Oxygen and CO<sub>2</sub> partial pressures and pH of blood and mock EDF were measured using a Radiometer PHM 71 acid-base analyzer and associated electrodes maintained at the same temperature as the fish (14–16°C). The pH electrode was calibrated with Radiometer standard pH buffers; the oxygen electrode was calibrated with air-saturated water and a Radiometer zero  $P_{O_2}$  solution; the CO<sub>2</sub> electrode was calibrated with precise gas mixtures from Wösthoff gas-mixing pumps. The CO<sub>2</sub> meter was adjusted to give full-scale readings for samples ranging from 0 to 2.67 kPa; consequently, precise measurements of mock EDF equilibrated with 3% or 5% CO<sub>2</sub> were not possible with our measuring system. We did, however, inject several samples of CO<sub>2</sub>-equilibrated mock EDF onto the CO<sub>2</sub> electrode. In each case the measurement indicated a  $P_{CO_2}$  value of 2.67 kPa, and most samples were off the scale of the meter. We are confident, therefore, that our calculated CO<sub>2</sub>-equilibrated solutions produced an adequate physiological stimulus (see Discussion). A blood sample was taken from the dorsal aortic (DA) cannula before an experiment began in normoxia or hypoxia for  $P_{O_2}$ ,  $P_{CO_2}$  and pH determination. The DA and buccal cavity cannulae were then attached to Statham P23Db and Hewlett-Packard 267BC pressure transducers, respectively, to monitor blood and buccal pressures. The outputs of the transducers were recorded on a Gulton Techni-rite (model 722) two-channel chart recorder. A 2 min record of DA and buccal pressures was taken as the pre-perfusion baseline of these variables. Perfusion was then begun by switching a three-way stopcock which connected the perfusate to the inflow cannula attached to the animal's cranium. The mock EDF flowed through the meningeal space for 30 min while DA and buccal pressures were recorded from 0 to 6 min, then at 2 min intervals encompassing the 10, 15, 20, 25 and 30 min post-perfusion time periods; therefore, total recording time for each protocol was about 18 min. The perfusate flow through the meningeal space varied between 1 and 3 ml min<sup>-1</sup> and was maintained by adjusting the pressure head between the inflow and outflow cannulae; this pressure differential ranged between 0.49 and 2.5 kPa and had no apparent effect on resting cardiovascular or ventilatory variables. Normoxic water ( $P_{O_2}$  20.7–21.3 kPa) flowed continuously through the Perspex holding box at a rate of about 1000 ml min<sup>-1</sup>. Fish were allowed 30–60 min recovery time between perfusate treatments until all four treatments were completed. Each experiment began with the normoxic (air-equilibrated) perfusate, but the other three perfusates were presented in random order. The fish were allowed to recover overnight and the

experiment was repeated with hypoxic water ( $P_{O_2}=4.0\text{--}5.3\text{ kPa}$ ) flowing through the Perspex holding box.

Experiments were also performed in which sodium cyanide (NaCN) dissolved in mock EDF at initial concentrations ranging from 5 to  $1000\ \mu\text{g ml}^{-1}$ , or hydrochloric acid (HCl) with pH ranging from 3.6 to 7.0, were added to the mock perfusate with the fish in normoxic water. Although the final pH was greater than the initial pH owing to dilution of  $\text{H}^+$  in the cranium, the final  $\text{H}^+$  concentrations measured were one or two orders of magnitude higher than those used to stimulate chemoreceptors in mammals and turtles (Shams, 1985; Hitzig and Jackson, 1978). NaCN is a metabolic inhibitor of the mitochondrial electron transport chain and is known to stimulate all  $\text{O}_2$ -sensitive chemoreceptors, including carotid body  $\text{O}_2$  chemoreceptors in mammals (Mulligan and Lahiri, 1981) and  $\text{O}_2$ -sensitive chemoreceptors in fish gills (Burlison and Milsom, 1990). NaCN at these initial concentrations has been used successfully to localise  $\text{O}_2$ -sensitive chemoreceptors in other fish (Smatresk, 1986; Smatresk *et al.* 1986; Burlison and Smatresk, 1990).

After an experiment was completed, the fish was killed by over-anesthetisation with concentrated MS 222. Sudan Black dye dissolved in 95 % ethanol, which has previously been used as a neural stain (Filipski and Wilson, 1984), was perfused through the meningeal space for 30 min with the same pressure head as during the experiment. After staining, fresh ethanol was perfused through the space to rinse out the excess dye. The brain was then removed and examined for the presence of dye. The extent of staining was taken to indicate where the mock perfusate had come into contact with CNS structures during the experiment. Unless otherwise stated, values are reported as mean  $\pm$  standard error. Differences between mean values of ventilatory frequency, buccal pressure, DA pressure and heart rate were compared using a three-level nested analysis of variance (ANOVA) with time, perfusate type and individual fish as covariates (Sokal and Rohlf, 1981). Blood gas data was compared during aquatic normoxia and hypoxia using a paired *t*-test. Air-breathing rates during aquatic hypoxia were compared to normoxia (zero air-breaths) using a one-tailed *t*-test.

## Results

Measured EDF concentrations ( $\text{mequiv l}^{-1}$ ) of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$  were similar to those found in other actinopterygian fishes:  $\text{Na}^+=116.0\pm 3.9$  ( $N=11$ ),  $\text{Cl}^-=123.6\pm 2.5$  ( $N=15$ ),  $\text{K}^+=1.2\pm 0.10$  ( $N=11$ ).

Gill ventilation rate, buccal pressure amplitude and DA pressure increased significantly ( $P<0.001$ ) with aquatic hypoxia compared with aquatic normoxia. Heart rate was unaffected by the hypoxic treatment. There was also no significant effect of perfusion time or perfusate treatment on any of the ventilatory or cardiovascular variables (Table 1); therefore, we present only the pre-perfusion and 15 min time interval data in Table 1. There were no recorded air-breaths during aquatic normoxia at this temperature (Tables 1, 2). Air-breathing occurred during aquatic hypoxia only and was observed during each type of perfusion

Table 1. Summary of measured cardiovascular and ventilatory variables during intracranial perfusion with mock EDF equilibrated with four different gas mixtures under aquatic normoxic or hypoxic conditions

Perfusate	Aquatic normoxia				Aquatic hypoxia				
	Variable	Pre-perfusion	15 min post-perfusion	AB	Perfusate	Variable	Pre-perfusion	15 min post-perfusion	AB
Normoxia (N=8)	<i>f<sub>G</sub></i>	9.8±1.3	9.8±1.0	0	Normoxia (N=7)	<i>f<sub>G</sub></i>	16.4±1.5	16.1±1.8	3.2±0.9
	<i>P<sub>B</sub></i>	59±9.8	68.6±9.8	0		<i>P<sub>B</sub></i>	98.1±19.6	78.4±19.6	
	<i>f<sub>H</sub></i>	24.7±2.0	25.2±2.2	0		<i>f<sub>H</sub></i>	27.6±1.2	29.2±0.9	
	<i>P<sub>DA</sub></i>	2.45±0.10	2.49±0.10	0		<i>P<sub>DA</sub></i>	2.46±0.16	2.46±0.16	
Hypoxia (N=8)	<i>f<sub>G</sub></i>	11.8±1.1	11.1±0.9	0	Hypoxia (N=7)	<i>f<sub>G</sub></i>	17.6±1.8	16.7±1.6	3.2±0.9
	<i>P<sub>B</sub></i>	68.6±9.8	68.6±9.8	0		<i>P<sub>B</sub></i>	98.1±19.6	98.1±19.6	
	<i>f<sub>H</sub></i>	26.7±1.9	27.5±1.7	0		<i>f<sub>H</sub></i>	28.6±0.8	29.2±0.9	
	<i>P<sub>DA</sub></i>	2.64±0.16	2.56±0.13	0		<i>P<sub>DA</sub></i>	2.36±0.2	2.37±0.20	
Hypercapnia* (N=8)	<i>f<sub>G</sub></i>	11.2±0.9	11.6±1.1	0	Hypercapnia* (N=8)	<i>f<sub>G</sub></i>	18.1±1.7	18.5±1.6	2.4±0.9
	<i>P<sub>B</sub></i>	58.9±9.8	68.6±9.8	0		<i>P<sub>B</sub></i>	108±19.6	98.1±19.6	
	<i>f<sub>H</sub></i>	27.4±1.5	27.8±1.5	0		<i>f<sub>H</sub></i>	28.7±1.0	28.2±1.3	
	<i>P<sub>DA</sub></i>	2.33±0.1	2.35±0.07	0		<i>P<sub>DA</sub></i>	2.67±0.18	2.68±0.18	
Hyperoxia (N=7)	<i>f<sub>G</sub></i>	10.3±1.2	9.7±1.1	0	Hyperoxia (N=8)	<i>f<sub>G</sub></i>	18.8±1.6	18.3±1.7	3.1±1.0
	<i>P<sub>B</sub></i>	58.9±9.8	58.9±9.8	0		<i>P<sub>B</sub></i>	108±19.6	108±19.6	
	<i>f<sub>H</sub></i>	25.9±1.4	26.9±1.6	0		<i>f<sub>H</sub></i>	29.2±1.1	30.4±0.9	
	<i>P<sub>DA</sub></i>	2.37±0.15	2.48±0.13	0		<i>P<sub>DA</sub></i>	2.66±0.08	2.58±0.12	

\* Three fish were perfused with 3% CO<sub>2</sub> perfusate and five fish were perfused with 5% CO<sub>2</sub> perfusate.

Data are presented here for pre-perfusion and at 15 min post-perfusion.

The measured variables are gill ventilation rate (*f<sub>G</sub>*; breaths min<sup>-1</sup>); buccal pressure amplitude (*P<sub>B</sub>*; Pa); heart rate (*f<sub>H</sub>*; beats min<sup>-1</sup>) and dorsal aortic blood pressure (*P<sub>DA</sub>*; kPa).

Air-breathing rates (AB; breaths h<sup>-1</sup>) for each treatment are also listed.

Values are means±s.e., *N* is the number of animals.

Table 2. Summary of dorsal aortic  $P_{O_2}$ , pH and  $P_{CO_2}$  and air-breathing rates (AB) during aquatic normoxia and hypoxia in *Amia calva*

Condition	$P_{O_2}$ (kPa)	pH	$P_{CO_2}$ (kPa)	AB (h <sup>-1</sup> )
Normoxia	9.12±1.6 (8)	7.74±0.02 (8)	0.4±0.03 (7)	0 (8)
Hypoxia	2.57±0.25 (8)	7.81±0.03 (6)	0.19±0.01 (8)	3.0±0.6 (8)
Significance ( <i>P</i> )	<0.005	<0.02	<0.001	<0.001

Values are mean±s.e. (*N*).

(Table 1). Air-breathing rates for each treatment were calculated by dividing the total air-breaths during a treatment by the total minutes recorded for a treatment; these values were converted to breaths h<sup>-1</sup> and are presented in Table 1. Air-breathing was not affected by perfusate treatment (ANOVA,  $F_{3,26}=0.15$ ;  $P>0.05$ ). The rate of air-breathing during aquatic hypoxia, with all perfusate treatments included, was 3.0±0.6 breaths h<sup>-1</sup> ( $N=8$ ) and was significantly different from zero ( $T_7=5.0$ ;  $P<0.001$ ).

The cardiovascular and ventilatory responses to intracranial NaCN were more variable. NaCN increased ventilation and DA pressure in one fish at a concentration of 500 µg ml<sup>-1</sup> and increased ventilation in another fish at a concentration of 1000 µg ml<sup>-1</sup>, but had no effect at lower doses. NaCN depressed ventilation in three other fish at concentrations ranging from 5 to 500 µg ml<sup>-1</sup>. Ventilation was unaffected in five fish perfused intracranially for 30 min with HCl solutions ranging in pH from 3.8 to 6.8.

Sudan Black dye stained extensive areas of the brain and associated structures. Dye was found occasionally within the third ventricle and usually in the fourth ventricle. Dye was always present on structures surrounding the brain, including the ventral surface of the medulla, cerebellum, optic tectum and telencephalon.

### Discussion

Central chemoreceptive loci, sensitive to CO<sub>2</sub> and/or pH, in terrestrial vertebrates are known to include the ventrolateral region of the medulla oblongata (Mitchell *et al.* 1963), and to be accessible from the ventricular system (Pappenheimer *et al.* 1965; Hitzig and Jackson, 1978). In this study, the CSF was not manipulated directly but, instead, mock EDF was perfused throughout the meningeal space. In fishes, the meningeal space lies between the periosteum of the cranium and the meninx overlying the brain surface; this communicates through blood vessels with the ventricular system and CSF. This has been confirmed using radioactive tracer techniques and vital dyes (see Davson, 1967). The dye perfusion results in this study indicate that our method of cranial perfusion resulted in the mock EDF coming into contact with areas of the brain corresponding with central

reflexogenic areas of mammals (Pappenheimer *et al.* 1965; Mitchell *et al.* 1963), turtles (Hitzig and Jackson, 1978) and toads (Smatresk and Smits, 1989). The lack of ventilatory or cardiovascular effects in response to intracranial perfusions of hypercapnic and low-pH solutions would indicate that *Amia* do not possess central chemoreceptive sites analogous to those that modulate ventilation in terrestrial vertebrates.

The ventilatory responses observed in *Amia* during aquatic hypoxia are quantitatively similar to those found by Johansen *et al.* (1970) at the same temperature and level of oxygenation. These observations indicate that our perfusion arrangement did not affect normal ventilatory functions in these animals.

Given that putative central chemosensitive sites were perfused with solutions containing physiological stimuli in animals exhibiting normal ventilatory responses to aquatic stimuli, the data indicate that *Amia* also lack centrally mediated chemoreflex responses to hypoxia. In support of this conclusion, experiments using isolated, spontaneously breathing carp heads reveal that respiratory movements are depressed, and eventually cease, when vascular perfusion is stopped (Kawasaki, 1980). This suggests that central hypoxia depresses, rather than stimulates, ventilation in isolated preparations and indirectly supports the suggestion that fish do not possess central O<sub>2</sub>-sensitive chemoreceptors.

Suggestions that central O<sub>2</sub> chemoreceptors exert control over gill ventilation are based only upon indirect evidence from water-breathing fish. Bamford (1974) showed that the ventilatory response time to aquatic hypoxia in rainbow trout was about 5 s, which he felt was a delay longer than would be necessary for stimulation of peripheral (i.e. gill) sites. Furthermore, at least two studies have shown that hypoxic reflexes persist after complete section of the glossopharyngeal and vagus nerves in tench (Hughes and Shelton, 1962) and sea raven (Saunders and Sutterlin, 1971). These nerves are believed to carry all chemoreceptor afferent fibres from the gills. One possible explanation for the failure to abolish hypoxic reflexes in tench and sea raven is that chemosensory information may also be conveyed through the facial nerve (cranial nerve VII). The pseudobranch of trout, which has been shown to possess some chemosensitivity to hypoxia and low-pH solutions (Laurent and Rouzeau, 1972), is innervated by the facial nerve in some species (see Nilsson, 1984). In channel catfish, a teleost which lacks a pseudobranch, the ventilatory responses to hypoxia or NaCN are abolished by complete denervation of the glossopharyngeal and vagal branches supplying the gills (Burleson and Smatresk, 1986). Furthermore, peripheral O<sub>2</sub>-sensitive chemoreceptors affecting ventilation have been demonstrated in air-breathing fish (lungfish, Lahiri *et al.* 1970, and gar, Smatresk, 1986; Smatresk *et al.* 1986).

The lack of significant effects on cardiovascular and ventilatory function during intracranial perfusions in this study argues against the hypothesis of central chemoreceptor involvement of either O<sub>2</sub> or CO<sub>2</sub>/pH in the ventilatory control of *Amia*; if central chemoreceptors do exist in this species, they must be insensitive to changes in the EDF composition around the brain.

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