Effects of hypoxia, anoxia, and endogenous ethanol on thermoregulation in goldfish, Carassius auratus

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Rausch, Richard N., Larry I. Crawshaw, and Helen L. Wallace. Effects of hypoxia, anoxia, and endogenous ethanol on thermoregulation in goldfish, Carassius auratus. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R545–R555, 2000.—Effects of hypoxia, anoxia, and endogenous ethanol (EtOH) on selected temperature (Tsel) and activity in goldfish were evaluated. Blood and brain EtOH concentrations ([EtOH]) and brain oxygen partial pressure (P O2) were quantified at crucial ambient oxygen pressures. Below a threshold value near 31 Torr, Tsel decreased as a function of environmental P O2. Tsel of 15°C-acclimated fish was −10°C at the onset of anoxia and changed little over 2 h. Activity showed a similar response pattern. Brain [EtOH] was significantly elevated above control levels after 1 h anoxia. In normoxic water, T sel remained different in previously anoxic and normoxic control fish for ~20 min. Blood [EtOH] of previously anoxia fish remained significantly elevated ([EtOH] >4.0 µmol/g blood), and activity was significantly depressed at 20 min. Brain P O2 reached normal levels in ~3 min. We conclude that [EtOH] (brain or blood) and brain P O2 are not proximal causes of either behavioral anaptyrexia (hypothermia) or inactivity in goldfish exposed to oxygen-depleted environments.

alcohol; temperature selection; fish; behavioral thermoregulation

SEVERE HYPOXIA LEADS TO DECREASES in core body temperature (T c). In thermal choice experiments, concomitant declines in selected temperature (T sel) and in T c indicate that hypoxia induces a decrease in the regulated temperature (anaptyrexia). Hypoxia has been demonstrated to elicit anaptyrexia in fresh- and saltwater fishes, amphibians and reptiles (4, 13, 18, 30), as well as crustaceans and protozoans (13, 22). Similar responses are also seen in endotherms such as rats and mice (14, 17). Physiological responses have also been used to demonstrate anaptyrexia. Under hypoxia, several lizard species activate a heat-loss response (gaping) at lower skin and core temperatures than under normoxia (18).

The degree of hypoxia required to elicit anaptyrexia varies across species. Significant decreases in the T sel of Plains minnows (4) are seen at an ambient oxygen partial pressure (P O2) below 110 Torr (1 Torr = 133.3 Pa), whereas the critical P O2 for anaptyrexia in Atlantic cod (30) lies between 32 and 47 Torr. Thermoregulatory responses to hypoxia are clearly linked to blood oxygen capacity because experimentally induced decreases in hematocrit lead to decreases in T sel in both reptiles and amphibians (18, 38). Responses may also reflect variations in the ability to mobilize physiological defenses against hypoxia, which is accomplished in numerous ways in different species (19). Three fishes of the family Cyprinidae greatly extend their survival in anoxic water by metabolizing lactic acid to ethanol (EtOH), which is then eliminated by diffusion across the gills (20, 31, 34).

Exogenously administered EtOH affects T c and T sel in many species (10). For goldfish, both EtOH immersion (25) and intrahypothalamic EtOH microinjections (11) decrease T sel. The concentration of EtOH ([EtOH]) required to elicit the behavioral response by intrahypothalamic microinjection is similar to the endogenously produced [EtOH] found in anoxic goldfish (31).

The goldfish was the subject of this study. Behavioral thermoregulation in this species proved both continuous and accurate. The ability of this species to remain functional under severely hypoxic and anoxic conditions allowed a complete delineation of the functional relationship among ambient P O2, T sel, and activity. A temperature choice device and aquatic thermal gradients were employed in separate experiments to quantify behavior patterns that occurred over a few hours in response to progressive hypoxia, anoxia, and ambient reoxygenation. Behavioral changes lasting only minutes during the transition from anoxia to normoxia were further characterized by maintaining anoxic animals under temperature-controlled conditions and subsequently transferring them directly to normoxic temperature gradients.

We examined two hypotheses for the proximal stimulus that leads to a decrease in T sel. One was that a sensed decrease in P O2 of the brain or some other tissue initiates a change in T sel. A second hypothesis was that anoxia-induced EtOH production in muscle increases brain EtOH concentration ([EtOH]brain), which decreases T sel. Fish were exposed in a controlled environment to the same temporal relationship between T sel and ambient P O2, as that observed in the behavioral experiments. Brain P O2 and tissue [EtOH] from these fish were correlated with behavioral responses.
MATERIALS AND METHODS

Animals. Goldfish (Carassius auratus) from 9 to 69 g were acclimated to 25°C (12:12-h light-dark photoperiod, indoors) for experiment 1 or to 15°C (natural photoperiod, greenhouse) for the remaining experiments. Acclimation was for a minimum of 2 wk in 120-l plastic aquaria. Water used for maintenance and experimentation was dechlorinated and treated with NaCl and KI (10:1) to elevate total salt concentration to 2.8 mg/l. Fish were fed commercial fish flakes daily but were not fed within 12 h of an experiment.

On the day of an experiment, a fish was placed into a tank at 25°C. Ten minutes later, flow was initiated from the hot reservoir. Data collected during the next 1 h were used to determine a normoxic $T_{sel}$ (mean ± SE) for an individual fish on a given experimental day. Next, water PO$_2$ was adjusted to the desired level. $T_{sel}$ was then monitored for at least 30 min. Water PO$_2$ varied slightly during this period and is reported as mean ± SE. Some fish were tested at more than one level of hypoxia during an individual experiment. For purposes of analysis, one data point was defined as the difference between mean T$_{sel}$ under hypoxic and normoxic conditions observed for an individual fish on a given experimental day [change in T$_{sel}$ ($\Delta T_{sel}$) = hypoxic T$_{sel}$ − normoxic T$_{sel}$]. Negative values, therefore, indicate a decrease in T$_{sel}$.

Because sample variances were unequal, a Kruskal-Wallis one-way ANOVA on ranks was used to evaluate the overall effect of environmental PO$_2$ on $\Delta T_{sel}$. The protocol analysis program of Yeager and Ultsch (41) was used to estimate the critical point at which decreasing ambient PO$_2$ began to exert a significant effect on T$_{sel}$.

Protocol adjustments. We employed two different devices to assess the effects of ambient oxygen pressure and endogenously produced ethanol on thermoregulatory behavior in goldfish. Two different acclimation temperatures were also used in the experiments. [EtOH] was quantified in brain or blood tissues in different experiments. The rationale for employing particular procedures follows.

A 25°C acclimation temperature was used in experiment 1 because it falls within the range of values reported as the final thermal preferendum for goldfish (6). The final thermal preferendum is the narrow, species-specific range of temperatures that undisturbed animals eventually select when exposed to a thermal gradient for a long period of time. By definition, when the final thermal preferendum is reached, acclimation temperature and T$_{sel}$ are the same. Thermal history (e.g., acclimation temperature) affects T$_{sel}$ in the short term. For example, animals acclimated to 25°C and placed into an aquatic thermal gradient, brown bullheads acclimated to 7, 15, 24, or 32°C selected 16, 21, 26, and 31°C, respectively. Within 1 day, however, all fish selected 29–31°C water (7). Acclimating fish to their final thermal preferendum prior to experimentation simplifies experimental design and interpretation of investigations involving behavioral thermoregulation.

The design of the temperature choice device (described above) was well suited to experiment 1. The PO$_2$ of a small volume of water could be changed rapidly and maintained at a stable level. However, the device was unsuitable for examining behavioral thermoregulation in anoxic goldfish. In pilot experiments, fish responded to environmental anoxia by initiating a tank temperature decrease and then becoming quiescent. This lack of activity allowed water temperature to fall to ~2°C. Fish acclimated to 25°C lost equilibrium under these conditions. If moved into the warm side of their tank, fish reacted to their new environment by shifting to the cold side recess, swimming rapidly, and then stopping if the investigator, fish quickly recovered as water temperature increased. Immediately following recovery, however, fish returned to the cold side recess and thus initiated tank cooling. Fish again became quiescent as water temperature decreased and soon lost equilibrium at low temperature.

To quantify responses during anoxia, two procedural adjustments were made. First, fish were tested in experiment 2 and in all subsequent experiments were acclimated to 15 rather than...
25°C. Biochemical adaptation allows the fish to better tolerate low (selected) temperatures (15). Second, thermoregulating fish were exposed to anoxia in an array of nine aquatic temperature gradients (3–31°C). In contrast to the temperature choice device, fish are not forced to remain active in an aquatic gradient.

Clarifying the role of endogenously produced EtOH in anoxic anapyrexia required the analysis of tissues obtained from anoxic fish. However, the large size of the apparatus (245 × 185 cm; water depth 9 cm) and the necessity for covering the entire array to maintain anoxic conditions made removing individual thermoregulating fish directly from the gradient array impracticable. Furthermore, removing one fish from its gradient would have disturbed other fish in nearby gradients.

The gradient array and small (6 liter) chambers were therefore used in a complementary manner to correlate thermoregulation with tissue EtOH levels. First, the time course of thermoregulatory effects of sequential exposure to periods of increasing hypoxia, anoxia lasting up to 2 h, and ambient reoxygenation was determined for fish in the gradient array (experiments 2 and 4). The small chambers were used in a second pair of experiments (experiments 3 and 5). Water temperature and PO2 in the chambers were manipulated remotely to reproduce the temporal relationship between these variables observed in the gradient experiments. Tissue samples for EtOH analysis were obtained at critical periods from fish exposed to this controlled environment.

Finally, to correlate EtOH production with the behavioral effects of oxygen depletion, [EtOH] was assessed in two different tissues. First, [EtOH]brain was quantified (experiment 3) to establish the relationship between central nervous system EtOH levels and the behavioral changes that occurred over a period of hours in the gradients (experiment 2). For a more detailed characterization of EtOH levels during behavioral transitions that lasted only a few minutes (experiment 4), the relative rapidity with which blood samples can be obtained made that tissue more suitable for analysis (experiment 5).

Blood [EtOH] ([EtOH]blood) is an appropriate index of central nervous system [EtOH]. Shoubridge and Hochachka (31, 32) established that EtOH fermentation occurs in goldfish red and white muscle, but not in other tissues. EtOH, therefore, diffuses from muscle to blood and from blood to brain and other organs where it can be metabolized. Unmetabolized EtOH diffuses across the gill epithelium into ambient water. [EtOH]blood, therefore, remains higher than [EtOH]brain. We validated and quantified this relationship for our assay method.

Experiment 2: effects of anoxia and anoxia duration on Tsel and activity. The thermoregulatory effects of anoxia were investigated using 15°C-acclimated goldfish exposed to aquatic thermal gradients (3–31°C). Training involved placing fish in a gradient for 4 h. Nylon mesh barriers prevented the fish from entering water warmer than 18°C. The barriers were necessary to minimize the selection of warm water, which rapidly initiates warm acclimation.

Positions of individual fish in each lane of an array of aquatic thermal gradients (245 × 185 × 9 cm) were recorded every 6 s. An image from a wide-angle camera above the gradients was digitized by an Oculus frame grabber (Coreco), processed by application-specific software, and stored on a Zenith 386 computer. Water temperature was measured at 10 points along each gradient and was used to transform position information into Tsel values (25). In addition, the absolute value of the distance moved (cm) by each fish during each 6-s interval was used as an index of activity.

A nonglare Plexiglas top and Visqueen plastic sides were fitted over the gradient array to facilitate manipulation of water PO2. Plexiglas lids sealed ports that provided access to 15°C water in each gradient. A PO2 probe was calibrated at 15°C in air- and N2-aerated water as in experiment 1 and positioned in 15°C water in one of the gradients.

Because the rate of PO2 decrease in the gradient was not rapid, hypoxia exposure was begun in jars to minimize the choice of and acclimation to warm temperatures during the initial period in the gradient. Pairs of fish were placed into 3.8-liter glass jars containing 15°C water, and nitrogen gas-aeration of the jars and the gradient array was scheduled so that water PO2 in both reached 45 Torr at the same time. Approximately 20 min were required to lower PO2 in the jars to this level. Fish were then quickly removed from the jars and placed, via the ports, one to a gradient, in 15°C water in the gradient array. Temperature selection was monitored as continued aeration with N2 decreased gradient water PO2 to 0 Torr, which was maintained for periods of up to 2 h thereafter when air-aeration was initiated. Water PO2 was recorded every 2 min.

Mean Tsel and activity levels of goldfish under severely hypoxic and anoxic conditions in the gradients were calculated for successive 10-min intervals. One fish died in the warm end of its gradient and was not included in the analysis. ANOVA with repeated measures was used to examine PO2 effects on Tsel. The effects of oxygen pressure on activity during anoxia and reoxygenation were evaluated using one-way repeated-measures ANOVA. Because sample variances were unequal, activity during progressive hypoxia was evaluated with Friedman repeated-measures ANOVA on ranks.

Experiment 3: brain EtOH concentration as a function of ambient PO2. The effects of PO2 on [EtOH]brain during progressive hypoxia and anoxia were investigated using small (6 liters) aquatic chambers, each of which contained a single fish. This was done to permit the correlation of central nervous system EtOH levels with thermoregulatory behavior. Water temperature and PO2 in the chambers were manipulated to mimic as closely as possible the temporal relationship between PO2 and Tsel observed in experiment 2. Aeration with pure N2 was begun and the temperature of the chambers adjusted as follows: 0–15 min: 15°C; 15–25 min: 19°C; 25–35 min: 17°C; 35–45 min: 15°C; 45–65 min: 14°C; 65–95 min: 12°C; 95 min: 9°C. Water PO2 was recorded every 2 min.

Fish were removed from the chambers at intervals and killed with a blow to the head. Brains were quickly removed and immediately placed in preweighed, 1.5-ml Eppendorf microcentrifuge tubes containing 1.0 ml TCA (6.25% wt/vol). The tubes were reweighed to determine brain weights. The samples were homogenized with a Teflon pestle, agitated with a vortex mixer, and centrifuged at 2,000 rpm for 5 min. A commercial EtOH assay (Sigma #332-UV) was used to quantify [EtOH]brain. A 0.5-ml sample of supernatant was added to NAD+/NADH reaction vials containing 2.0 ml glycine buffer (0.5 M) and incubated at room temperature for 10 min. Control reaction vials received 0.5 ml TCA. Absorbance at 340 nm was measured using a Coleman 620 spectrophotometer. Calculation of [EtOH]brain (µmol/g tissue) was from a previously prepared standard curve.

Approximately 10 min were required to extract the brains of all fish in the chambers and transfer the samples to the TCA solution. Because PO2 was falling rapidly during the first 70 min of N2 aeration, a mean PO2 based on 2-min sampling intervals was taken as indicative of the degree of hypoxia experienced by the fish during this period. For longer-lasting experiments, the PO2 at the beginning of the extraction period was used because PO2 had stabilized at extremely low levels.
The effects of hypoxia, anoxia, and anoxia duration on [EtOH]_{brain} were analyzed using one-way ANOVA. Multiple comparisons were made with the Tukey test.

Experiment 4: temperature selection and activity following anoxia. The effects of a rapid, single-step exposure to normoxic water on T_{sel} and activity in previously anoxic goldfish were quantified in aquatic thermal gradients (4–32°C). Before being placed in the gradients, four or five fish were housed for 4 h in one of two 3.8-liter jars containing 15°C water and 25 g Ammonex. Water in the first jar was made anoxic by aeration with N2 for the first hour. This jar was then sealed and placed in a 15°C, 120-liter tank for 3 h. Water in the second jar was aerated with air during the first hour. During the next 3 h, a plastic mesh lid allowed aerated tank water to enter the jar.

Fish from both jars were placed individually at 15°C into normoxic temperature gradients. Thermoregulatory behavior was monitored for at least 1.5 h. Mean T_{sel} during 2-min intervals was calculated for each fish. Of 36 fish tested in this manner, one fish died and two lost equilibrium; these fish were excluded from the data analysis.

Two-way ANOVA with repeated measures (on ranks when variances were unequal) was used to evaluate the effects of treatment (anoxic or normoxic) and time in the gradient on T_{sel} and activity. A two-tailed t-test was used to identify the time period during which the difference in mean T_{sel} between groups was maximal.

Experiment 5: EtOH elimination following anoxia. If anoxia-induced [EtOH] production was responsible for the differences in T_{sel} observed in the early stages of experiment 4, EtOH levels should be similar when T_{sel} of the two groups of fish were the same. To determine the time course for changes in [EtOH]_{blood} that occurs when previously anoxic (3 h) fish are exposed to a normoxic environment, goldfish were injected with heparinized 0.7% saline (3.5% of body wt) and placed, in pairs, into 3.8-liter glass jars containing 15°C water and 25 g Ammonex. The anoxic and normoxic conditions were created as in experiment 4.

Blood samples were obtained from fish immediately on removal from the jars or after 6- or 22-min exposure to normoxic 15°C water. Fish were killed with a blow to the head, tails were excised, and blood samples were collected rostral to the excision in heparinized capillary tubes. An alternative procedure for the 22-min sampling interval was also employed. The temperature of the aerated water was adjusted to approximate the mean T_{sel} observed in experiment 4 as follows: 0–6 min: 15°C; 6–18 min: 18°C; 18–22 min: 20°C. Blood samples were analyzed using the method described for experiment 3, except that the samples were not homogenized.

Blood EtOH sample variances were unequal so the effects of treatment (anoxic or normoxic) and time in normoxic water on [EtOH]_{blood} were evaluated using two-way ANOVA on ranks. Multiple comparisons within the anoxic group were made using the Tukey test. A two-tailed t-test was used to assess differences in [EtOH]_{blood} obtained using the different 22-min protocols.

Experiment 6: brain reoxygenation following anoxia. Goldfish were implanted with chronic guide cannulae that provided access to brain tissue for the in situ measurement of brain PO2. This was done to quantify the rate at which brain PO2 increased to normal levels following anoxia and to determine whether long- and short-term anoxia were different in this regard.

A guide cannula with a Teflon-coated silver reference electrode mounted on its outer wall was implanted in each fish following previously described stereotaxic procedures (36). A small puncture through the meninges allowed the tip of the guide cannula to be placed in contact with the caudal portion of the tectum. The chlorided end of the reference electrode was positioned at the same level as the cannula tip but did not contact either the cannula or the brain tissue. Vitallium bone screws and dental acrylic were used to affix the cannula to the surface of the skull. After surgery, a 23-gauge indwelling stylet with a rubber collar was inserted into the guide cannula. The tip of the stylet penetrated ~0.5 mm into the tectum. Fish were able to swim and feed normally within 20 min after surgery.

Brain PO2 was monitored using a bipolar oxygen electrode (AM Systems, model 5862) coupled to a Transidyne General chemical microsensor (model 1201), the output of which was recorded on a Linear chart recorder. A polyethylene tube (PE 90) within the guide cannula accepted the 23-gauge electrode and prevented the steel walls of the cannula from abrading the electrode tip. A collar constructed from stainless steel and rubber tubing held the electrode in place and prevented water leakage through the cannula. During experiments, PO2 probes were inserted so the electrode tip extended ~0.5 mm beyond the position formerly occupied by the end of the stylet.

To allow rapid alteration of water PO2, individual fish were placed in a chamber that measured 25.5 × 5.0 × 7.5 cm. The total volume of water in the chamber, including inflow and outflow tubes, was 0.8 liter. Water maintained at 15°C was pumped through the chamber from front to back in a circuit fed from either of two reservoirs. An adjustable barrier caused fish to face the inflow tube but did not interfere with opercular movements. The first reservoir was continually aerated with room air. Water in the second reservoir was anoxic due to N2 bubbling. Appropriate bubbling also produced a slight positive pressure within the chamber. The submersible circulation pump could be quickly transferred from one reservoir to another to effect a rapid change in the PO2 of the chamber water. Water from a second reservoir reached a fish’s head 11 s after the pump was moved. Water in this region was completely replaced <1 s later.

Prior to testing in the chamber, fish were housed in either a normoxic or an anoxic aquatic environment for at least 4 h. One group of fish was housed and instrumented under anoxic conditions. The chamber was divided into three segments by an adjustable barrier that created a flow of water from one reservoir to another to effect a rapid change in the PO2 of the chamber water. Water from a second reservoir reached a fish’s head 11 s after the pump was moved. Water in this region was completed replaced <1 s later.

A second group of fish was maintained in a normoxic reservoir, instrumented, and placed into the test chamber that received water from the same reservoir. Fifteen minutes later, or when a stable brain PO2 had been established, the circulation pump was moved to the anoxic water reservoir. Brain PO2 reached 0 Torr in 4–9 min (mean ± SE = 6.1 ± 1.1 min). The pump was then returned to the normoxic reservoir, and recording continued for a minimum of 4 min.

Three additional goldfish were implanted with cannulas, as described above, to ensure that changes in brain PO2, under the conditions of this experiment, were not the result of chamber water leaking into the cranium. First, a baseline PO2 was recorded for each fish in the chamber under normoxic conditions. The chamber pump was then transferred to a reservoir containing anoxic water. When brain PO2 reached 0 Torr, the pump was moved to a third reservoir containing anoxic water and MS-222 (175:1; pH adjusted to 7.0) for 6 min. Finally, the circulation pump was transferred to normoxic water and recording continued for at least 6.5 min thereafter. Pilot experiments showed that the hearts of fish exposed to this anesthetic concentration stopped beating within 5 min. Any increase in brain PO2 under these condi-
tions would, therefore, be due to cannula leakage and not to respiration by the fish.

The PO2 probe was calibrated in both air- and N2-aerated 0.7% saline at 15°C before and after each run. Significant drift was sometimes evident during postexperimental calibration of the probe in normoxic, but not anoxic, saline. The reading obtained 7 min after the probe was removed from the brain and placed into aerated saline was taken as the standard normoxic value and was used to calculate brain PO2.

The brain reoxygenation parameters used for comparison of chronically (4 h) and acutely (4–9 min) anoxic groups of fish were as follows: time to an initial increase in PO2, maximum postanoxic PO2 (PO2max), time to PO2max and PO2 change during the first 4 min. As an index of the rate at which PO2 increased, the time required to reach one-half of the 4 min PO2 was also calculated. Brain PO2 during the first 4 min exposure to normoxic water was compared between groups with a two-way ANOVA. Group values for the other parameters were compared using a two-tailed t-test or, when sample variances were unequal, the Mann-Whitney U-test.

### RESULTS

**Experiment 1: effect of hypoxia on Tsel and activity.** A record of Tsel during 30-s intervals for one trained goldfish in the temperature choice device under normoxic conditions is depicted in Fig. 1. In marked contrast to naive fish (not shown), trained goldfish did not allow normoxic water temperature to reach the upper or lower extremes attainable by the device. Fish did not constantly shuttle from one recess to another. Instead, most fish triggered a temperature change and soon moved back out into the main portion of the tank. The mean Tsel for this fish under normoxic conditions was 25.3 ± 0.2°C.

**Figure 1** also shows Tsel for the same fish under severely hypoxic conditions. The mean Tsel at a water PO2 near 8 Torr was 16.8 ± 0.8°C, 8.5°C lower than the normoxic Tsel. Both maximum and minimum water temperatures (estimated as upper and lower exit temperatures) were lower than those observed during normoxia. As PO2 was initially reduced, fish generally became agitated and often gulped surface water and air. At very low PO2, fish settled to the bottom of the tank and became quiescent.

![Graph](image)

**Fig. 1.** Representative temperature selection records for an individual goldfish in a temperature choice device under normoxia (●) and severe hypoxia (O, O2 partial pressure [PO2] = 8 Torr). Mean hypoxic selected temperature (Tsel) was −8.5°C lower than mean normoxic Tsel.

### Table 1. Upper and lower exit temperatures

<table>
<thead>
<tr>
<th>PO2, Torr</th>
<th>UET, °C</th>
<th>LET, °C</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>31.0 ± 0.3</td>
<td>22.1 ± 0.3</td>
<td>46</td>
</tr>
<tr>
<td>136.6 ± 2.7</td>
<td>31.2 ± 0.6</td>
<td>23.1 ± 1.1</td>
<td>4</td>
</tr>
<tr>
<td>130.6 ± 2.2</td>
<td>31.9 ± 0.7</td>
<td>22.5 ± 1.9</td>
<td>4</td>
</tr>
<tr>
<td>119.4 ± 2.0</td>
<td>32.9 ± 0.6</td>
<td>23.2 ± 1.0</td>
<td>8</td>
</tr>
<tr>
<td>75.2 ± 1.7</td>
<td>30.9 ± 0.3</td>
<td>22.0 ± 0.9</td>
<td>4</td>
</tr>
<tr>
<td>70.5 ± 0.9</td>
<td>30.3 ± 0.6</td>
<td>22.1 ± 1.0</td>
<td>4</td>
</tr>
<tr>
<td>54.6 ± 1.1</td>
<td>28.9 ± 1.5</td>
<td>21.6 ± 0.9</td>
<td>2</td>
</tr>
<tr>
<td>48.2 ± 0.8</td>
<td>30.2 ± 0.9</td>
<td>22.1 ± 2.1</td>
<td>4</td>
</tr>
<tr>
<td>35.0 ± 3.9</td>
<td>30.6 ± 0.9</td>
<td>20.3 ± 0.9</td>
<td>6</td>
</tr>
<tr>
<td>30.0 ± 3.4</td>
<td>31.0 ± 0.7</td>
<td>20.4 ± 1.4</td>
<td>6</td>
</tr>
<tr>
<td>15.0 ± 2.4</td>
<td>28.4 ± 1.2</td>
<td>16.0 ± 0.7*</td>
<td>7</td>
</tr>
<tr>
<td>10.0 ± 1.4</td>
<td>24.3 ± 0.8*</td>
<td>14.6 ± 0.5*</td>
<td>10</td>
</tr>
<tr>
<td>6.0 ± 0.9</td>
<td>25.7 ± 0.9*</td>
<td>12.8 ± 0.7*</td>
<td>9</td>
</tr>
<tr>
<td>&lt;5.9</td>
<td>26.1 ± 1.2*</td>
<td>13.0 ± 0.6*</td>
<td>7</td>
</tr>
</tbody>
</table>

Values are means ± SE or PO2 range. UET, upper exit temperature; LET, lower exit temperature. n = No. of animals. *Significant difference from corresponding normoxic value (P < 0.05).

Severely hypoxic fish usually moved only when triggering a rise or fall in tank temperature.

However, the longer period of time between maximum (or minimum) temperature values seen at 8 Torr compared with normoxia in Fig. 1 should not be interpreted as being due solely to inactivity by the fish. Rather, as tank temperature approached that of a heat exchange bath, the rate of temperature change necessarily decreased. Therefore, a longer period of time passed before the fish was forced to respond to a temperature extreme.

Environmental PO2 had a significant effect on both upper (F[13,107] = 12.662; P < 0.001) and lower (F[13,107] = 23.507; P < 0.001) exit temperatures of goldfish (11 fish, 46 runs, 75 observations) tested in the temperature choice device (Table 1). Upper and lower exit temperatures were significantly different from their normoxic values at PO2 below 15 and 25 Torr, respectively. Upper exit temperatures were lowest between 10 and 15 Torr and tended to increase slightly at lower PO2.

The mean Tsel for goldfish in the temperature choice device under normoxic conditions was 26.5 ± 0.3°C. Figure 2 shows group data for the relationship between PO2 and Tsel in goldfish. Normoxic values are not shown. Each data point represents the difference in mean Tsel (ΔTsel) for an individual fish on a given experimental day under hypoxic and normoxic conditions. Kruskal-Wallis one-way ANOVA on ranks indicated that the effect of PO2 on ΔTsel was significant [H(0.05;23) = 76.594; P < 0.001]. A consistent effect of ΔTsel was not evident until PO2 was reduced below 40 Torr. The breakpoint analysis of Yeager and Ultsch (41) yielded lines 1 and 2 in Fig. 2. These lines are described respectively by the equations y = 0.01x – 1.5 and y = 0.39x – 12.2. The midpoint approximation of the critical point (threshold) for the effect of PO2 on ΔTsel was 31.2 Torr.

If we assume a linear function for line 2, extrapolation to 0 Torr would predict that Tsel for anoxic 25°C-acclimated goldfish exposed to anoxia in the device would be −12°C lower than their normoxic Tsel. Such
fish, however, did not tolerate anoxia in the temperature choice device (see Protocol adjustments).

Experiments 2 and 3: effects of anoxia onset and duration on Tsel, activity, and brain EtOH concentration. Mean Tsel (±SE; n = 14) of 15°C-acclimated goldfish during successive 10-min intervals of exposure to increasing hypoxia, anoxia, and subsequent reoxygenation in aquatic temperature gradients is shown in Fig. 3. Also shown is the water PO2 at the start of a particular time interval. Two groups of fish were exposed to the gradient protocol on different days. The difference in the rates at which water PO2 rose and fell was not statistically significant, so Tsel and activity data were pooled.

Goldfish placed at 15°C into progressively hypoxic (initial PO2 ~45 Torr) aquatic temperature gradients selected temperatures near 10°C with the onset of anoxia, and Tsel did not change significantly during periods of anoxia lasting 90 (n = 6) or 130 (n = 8) min [F(13,132) = 0.0961; P = 0.489].

Reoxygenation of gradient water (Fig. 3) led to significant increases in Tsel [F(7,111) = 18.215; P < 0.001]. To illustrate the effect of reoxygenation, 40 min of data from the longer duration group and 10 min of data from the shorter duration group were removed to create the break in Fig. 3. (Neither Tsel nor activity changed significantly during these intervals.) Thus zero on the right side of the abscissa indicates the initiation of air aeration.

Fig. 2. Effect of ambient oxygen pressure on Tsel (11 fish, 46 runs, 75 observations). Ordinate axis is change in Tsel from normoxic value for an individual fish on a given day. Negative numbers indicate a decrease in Tsel. Midpoint approximation of critical point for anapnoea is 31.2 Torr.

Fig. 3. Effects of increasing hypoxia, anoxia, and reoxygenation on Tsel and activity during 10-min intervals in aquatic temperature gradients. Two groups of fish were exposed to ambient anoxia in temperature gradients for 90 or 130 min. No significant differences in behavior or in rates of PO2 decrease or increase were found, so data were pooled. Data points are means (±SE) for 14 animals. Error bars too small to show on ambient PO2 values. To coordinate data to illustrate effects of reoxygenation, 40 min of data from longer-duration group and 10 min of data from shorter-duration group are not shown (break in abscissa). Arrow indicates initiation of air aeration.

Fig. 4. A: Tsel (means ± SE) of previously anoxic (n = 16) and normoxic (n = 17) 15°C-acclimated goldfish exposed to normoxic temperature gradients. B: activity (means ± SE) in same groups of fish.
aeration for both groups of fish. When normoxic conditions were established in the gradients, fish selected temperatures that were quite similar to those selected by 15°C-acclimated control fish exposed to normoxic temperature gradients for 30 min (experiment 4, Fig. 4A).

Activity was affected by environmental PO2 in a manner similar to that of Tsel (Fig. 3). During the first 10-min interval when PO2 was decreasing from 45 Torr, activity was higher than, but not statistically different (t(0.05(2), 28) = 1.697; P = 0.101) from that of normoxic, 15°C-acclimated fish immediately after placement into normoxic thermal gradients (experiment 4, Fig. 4B). Activity levels decreased during progressive hypoxia [χ2(0.05(10)) = 100.402; P < 0.001], did not change during anoxia [F(12,132) = 1.069; P = 0.391], and then increased as gradient water was oxygenated [F(7,91) = 8.551; P < 0.001]. By the time PO2 stabilized above 140 Torr, activity levels were very similar to those observed in previously anoxic fish exposed to normoxic gradients for 30 min (experiment 4, Fig. 4B).

[EtOH]brain data (means ± SE; Fig. 5) were collected from 34 goldfish exposed to a PO2, temperature, and time regime closely approximating that encountered by the fish in the temperature gradients (experiment 2). One-way ANOVA revealed a significant effect of PO2 on [EtOH]brain [F(6,27) = 9.154; P < 0.001]. Multiple comparisons indicated that only the samples obtained after 1 and 2 h anoxia were significantly different from the normoxic control value.

Experiments 4, 5, and 6: temperature selection, EtOH elimination, and brain reoxygenation following anoxia. Figure 4A demonstrates the effect of previous environmental anoxia exposure at 15°C on behavioral thermoregulation in a normoxic temperature gradient. Data points shown are mean Tsel (±SE) during 2-min intervals for previously anoxic fish (n = 16) and normoxic controls (n = 17). Mean Tsel for both groups increased significantly with time in the temperature gradient [F(14,434) = 5.765; P < 0.001]. Although both groups were selecting temperatures >20°C at 22 min, the rate of change in Tsel up to this time was lower in previously anoxic fish than in normoxic controls [F(1,310) = 4.321; P < 0.046]. The difference between Tsel for the previous anoxic and normoxic groups was greatest at 6 min (t(0.05(2),31) = 2.420; P = 0.0216).

Activity levels in both groups increased with time in normoxic temperature gradients [F(14,434) = 6.515; P < 0.001]. Activity in the previously anoxic group remained lower throughout the 30 min period (Fig. 4B, [F(1,434) = 9.578; P = 0.004].

Changes in [EtOH]blood and brain PO2 following exposure of previously anoxic fish to normoxic water are illustrated in Fig. 6. [EtOH]blood was higher in previously anoxic fish (n = 19) than in normoxic controls (n = 10; F(1,25) = 102.331; P < 0.001). [EtOH]blood did not differ between the two anoxic 22-min protocols (t(0.05(2),6) = 2.447); these data were pooled. The effect of time in normoxic water on [EtOH]blood within the previously anoxic group was statistically significant [F(2,16) = 7.240; P < 0.006]. [EtOH]blood was lowest in the anoxic group after 22 min but remained well above control levels. Multiple comparisons within the anoxic group indicated that [EtOH]blood at 22 min was significantly different from the 6-min value [q(0.05,20,3) = 5.112; P < 0.05].

A linear regression model of the relationship between [EtOH]blood and brain PO2, constructed for our assay method was statistically significant [F(1,20) = 45.297; P < 0.001; r2 = 0.711] and is described by the equation [EtOH]brain = 0.196 + 0.436·[EtOH]blood. With the use of this model, the [EtOH]blood values determined at 0, 6, and 22 min correspond to brain [EtOH]s of 3.1 ± 0.5, 3.7 ± 0.2, and 2.2 ± 0.4 µmol/g brain, respectively. The calculated value for the 0-min sample is similar to the [EtOH] measured in brains of fish exposed to progressive hypoxia and 2 h anoxia (2.80 ± 0.66 µmol/g brain, experiment 3).

Figure 6 also demonstrates that brain PO2 (means ± SE) of fish subjected to either long-term (4 h, n = 6) or short-term (4–9 min, n = 5) anoxia rose rapidly from 0 Torr when fish were exposed to oxygenated water. The effect of time in normoxic water on brain PO2 was significant [F(1,80) = 2.61; P < 0.01]. Of the reoxygenation indices measured, none differed between groups. Figure 6 shows that the brains of the long-term group reoxygenated somewhat more rapidly, but the differ-
ence was not statistically significant \[F(7,80) = 2.86; P = 0.11].

After a lethal dose of MS-222, brain PO₂ of three anoxic fish did not change from 0 Torr during 6 min of exposure to normoxic water. Therefore, leakage of ambient water into the cranium via the cannulas did not affect the brain reoxygenation results.

DISCUSSION

The ability of goldfish to survive anoxia made it possible to quantify thermoregulatory responses over a broad range of environmental oxygen pressures. For well-trained goldfish acclimated to their final thermal preferendum (∼25°C), data from the temperature choice device depicted an extremely abrupt fall in Tsel (i.e., the critical point for anapyrexia) when the ambient PO₂ fell to ∼31 Torr. Slightly above this PO₂, there was almost no effect on Tsel, whereas slightly below it Tsel declined precipitously to temperatures as low as 12°C below the normoxic Tsel. This corresponded to a Tsel of ∼14°C.

The constancy of Tsel until a very low ambient PO₂ is reached indicates a strong priority for maintaining the body temperature at a particular level. In a number of fish species, the normoxic Tsel coincides with thermal optima for many physiological functions (9). The critical point appears to represent the PO₂ at which oxygen delivery to the tissues becomes seriously compromised. It would be expected that this point would vary between animals, and even in the same animal under different conditions.

Other hypoxia-resistant species respond to hypoxia in a manner similar to that of the goldfish. The critical point for anapyrexia in Atlantic cod (Gadus morhua) is between 32 and 47 Torr (30), whereas that of the juvenile alligator (Alligator mississippiensis) lies between 30 and 35 Torr (3). In contrast, animals that are less likely to encounter hypoxia are characteristically less hypoxia resistant and have higher critical points. Typical air-breathing ectotherms and endotherms exhibit a decrease in Tsel when exposed to an ambient PO₂ between 50 and 75 Torr (12, 13, 17, 18, 40).

A metabolic approach for estimating the point of compromised O₂ delivery has been used for many years. If ambient PO₂ is slowly lowered, at some point the O₂ consumption declines below the standard rate. The PO₂ at which this decrease begins has been termed the critical point, the level of oxygen dependency, or the incipient limiting level of oxygen. In general, metabolic and behavioral assessments of the critical point yield similar results. Fry and Hart (16) found a critical PO₂ in the high thirties for goldfish acclimated and tested at 25°C. For goldfish acclimated and tested at 20°C, the critical PO₂ was in the low thirties (1). At 15°C (acclimation and test temperature), the critical PO₂ fell to the high twenties (16). The critical PO₂ is higher when fish are forced to remain active (16).

Whereas the selection of low temperature does aid in the survival of severe hypoxia by depressing the metabolic rate, such acute temperature decreases create other stresses (see Ref. 8). This was reflected when severely hypoxic 25°C-acclimated fish in the temperature choice device initiated a decrease in tank temperature. Their ensuing inactivity led to rapid, continuous decreases in water temperature until the minimum attainable temperature was reached. Under these conditions, fish lost the ability to maintain equilibrium. When 15°C-acclimated goldfish were exposed to progressive hypoxia below the critical point in temperature gradients, Tsel decreased largely in parallel with decreases in PO₂. Under anoxic conditions, mean Tsel for 15°C-acclimated goldfish was ∼10°C and did not change significantly during 1- or 2-h anoxia exposure.

In contrast to fish in the choice device, goldfish in the temperature gradients were not incapacitated by environmental oxygen depletion. They responded to the onset of hypoxia with some hyperactivity, but as the PO₂ continued to fall, they became quiescent. This response is similar to that of the hypoxia-resistant bluegill (Lepomis macrochirus) and brown bullhead (Ictalurus nebulosus), but is different from that of trout (Oncorhynchus mykiss), a species less resistant to hypoxia, which remains agitated when water PO₂ is decreased (5). Reoxygenation of gradient water was followed by somewhat greater activity; but when both previously anoxic and control fish were selecting similar temperatures, activity remained depressed in previously anoxic fish. This suggests the possibility of different mediators of anapyrexia and hypoactivity.

A relationship between blood oxygen capacity and anapyrexia in ectotherms has been clearly demonstrated. Toads made anemic select lower than normal temperatures (38). When exposed to graded hypoxia, arterial saturation in toads was higher when maintained at 15°C, their hypoxic Tsel, than at 24°C, their normoxic Tsel (40). The present experiments indicate that brain PO₂ is not likely to be the proximal cause of anapyrexia or hypoactivity. The brain PO₂ of both chronically (4 h) and acutely (6–9 min) anoxic goldfish reached normal values within 3 min of exposure to normoxic water, but Tsel and activity of anoxic fish remained depressed for at least 15 and 30 min, respectively.

Ambient oxygen level affects metabolic rate. Heat production in anoxic goldfish is reduced to only 30% of the normoxic value (33). A clear link between metabolic depression and anapyrexia has been established. Tsel under normoxic conditions was dramatically reduced in both toads (2) and paramecia (21) treated with inhibitors of oxidative phosphorylation. In toads, anapyrexia appeared to be mediated by an effect on the central nervous system rather than via hypoxemia or hypercapnia (2). In the present experiments, the coincidence of normal brain PO₂ with depressed Tsel and activity also argues against central nervous system PO₂ being the proximal stimulus for the thermoregulatory effects of ambient oxygen pressure, at least during reoxygenation.

We tested the hypothesis that endogenously produced EtOH was a mediator of anapyrexia and hypoactivity in severely hypoxic and anoxic goldfish and that its persistence was related to the duration of postanoxia effects on behavior. The data do not support this
hypothesis with regard to $T_{sel}$. During progressive hypoxia in the temperature gradients, the fall in $T_{sel}$ was largely completed by the time [EtOH]$_{brain}$ began to increase. No significant changes in $T_{sel}$ occurred as [EtOH]$_{brain}$ continued to rise (to 2.2 $\pm$ 0.6 µmol/g) over the next 2 h. Thus [EtOH]$_{brain}$ has little or no effect on $T_{sel}$.

This interpretation was corroborated when previously anoxic goldfish with high blood levels of EtOH were placed in normoxic thermal gradients. The fish moved into successively warmer water until, after 22 min, $T_{sel}$ for the previously anoxic fish was identical to that of previously normoxic controls. At this time, however, [EtOH]$_{blood}$ of the previously anoxic fish was still quite high (4.3 $\pm$ 0.4 µmol/g). On the basis of the linear regression for the relationship between [EtOH]$_{blood}$ and [EtOH]$_{brain}$ constructed for our assay method, this [EtOH]$_{blood}$ corresponds to a calculated [EtOH]$_{brain}$ of 2.2 $\pm$ 0.4 µmol/g, a value quite similar to that measured directly from anoxic fish. The large decrease in $T_{sel}$ following low doses of EtOH microinjected into the nucleus preopticus periventricularis (11) may reflect only a local effect. When [EtOH] is increased for the entire brain, as would occur as a result of endogenous EtOH production, effects on other areas may act to oppose signals emanating from that nucleus.

Changes in activity levels during progressive hypoxia and prolonged anoxia essentially paralleled the effects on $T_{sel}$. Therefore, endogenous EtOH does not initiate hypoactivity under those conditions. However, after 22 min in normoxic water, reduced activity was coincident with higher than normal [EtOH]$_{blood}$ in previously anoxic fish. These experiments were not specifically designed to evaluate long-term recovery from anoxia-induced hypoactivity. Tissue [EtOH]$_{s}$ of samples obtained as activity returned to normal levels could be used to address this issue. This was not done. Further study will be required to clarify the role of EtOH in this behavior.

Several lines of evidence suggest a role for catecholamines in hypoxic anapyrexia. Acute hypoxia exposure decreased blood PO$_2$ and O$_2$ content and elevated blood pH and catecholamine levels in Atlantic cod (28). Bilateral transection of autonomic nerves to chromaffin tissue aggregations within the head kidney did not prevent the hypoxia-induced rise in norepinephrine, thus suggesting a direct effect of hypoxemia on norepinephrine release from this tissue. Because hypoxia led to blood alkalosis, an increase in blood H$^+$ concentration did not appear to be the stimulus for norepinephrine release.

Intrahypothalamic injections of both norepinephrine and dopamine elicit the selection of lower temperatures in goldfish (35, 36). Both $\alpha_1$ and $\alpha_2$ adrenoceptors, but not $\beta$ receptors, are involved in the anapyretic response to norepinephrine (37). Because $\alpha$-antagonists blocked the response to subsequent norepinephrine injection but did not affect thermoregulation when injected alone, the authors proposed that noradrenergic influences on $T_{sel}$ may be most important under severe environmental conditions (such as hypoxia or anoxia). Interestingly, brain concentrations of monoamine neurotransmitters in anoxia-resistant species are preserved during anoxia, whereas those of anoxia-intolerant species decrease significantly (24).

Several advantages accrue when animals select low temperatures as environmental oxygen is depleted. Oxygen demand decreases at lower temperatures (16). Moffitt and Crawshaw (23) found that the Q$_{10}$ for metabolic rate following acute temperature changes within the range of 13–25°C was 2.61 in the common carp, Cyprinus carpio. In the present experiments, goldfish exposed to an ambient PO$_2$ near 10 Torr selected temperatures ~10°C lower than under normoxic conditions. If a similar Q$_{10}$ is assumed for goldfish and carp, this behavior would reduce the oxygen requirement by >50%. By minimizing voluntary motor activity, goldfish take full advantage of this effect.

Lower temperature also decreases the half-saturation value for hemoglobin, thus increasing the animal’s ability to extract oxygen from the surrounding water (39). The cost of routine respiration in quiescent fishes is thought to be ~10% of standard metabolic rate (27). Improved ability to extract oxygen from the environment would allow gill ventilation rate and its associated metabolic costs to be minimized.

The adaptive value of anapyrexia under hypoxic conditions has been demonstrated for a number of species. For Atlantic cod, reduced ambient temperature extends the lower lethal limit of oxygen tension (30). Marine toads survived 24 h at 25 Torr when maintained at 15°C, their preferred temperature at this PO$_2$, but died when housed at 25°C, the normoxic preferred temperature (40). For juvenile alligators, progressive hypoxia to near 24 Torr at constant temperature led to decreases in O$_2$ uptake and arterial pH and increases in plasma lactate concentrations that were significant at 25 and 35°C. These deleterious effects were absent, however, in alligators maintained at 15°C, their preferred temperature at an ambient PO$_2$ of 24 Torr (3).

In summary, in a heterothermal environment, many organisms, including goldfish, select cooler than normal temperatures when ambient PO$_2$ decreases. The degree of hypoxia required to produce this response often has a critical oxygen pressure. Above the critical value, thermoregulation is unaffected; below it $T_{sel}$ declines. This critical value varies among species. A complete characterization of the mechanism(s) involved in this generalized response and identification of factors responsible for species differences await further study.

Perspectives

The functional significance of EtOH production in anoxic goldfish does not appear to include an effect on $T_{sel}$. The mechanism of anapyrexia in goldfish is likely similar to that occurring in organisms from many taxa when a critical ambient PO$_2$ is reached. The series of events leading to this behavior remains to be eluci-
dated, but suggestive evidence is available in the literature.

The protracted time course for the return to normal activity and \( T_{\text{set}} \) evident when anoxic fish are returned to a normoxic environment (experiment 4) argues for a blood-borne mediator of anapyrexia. Below a critical \( \text{PO}_2 \), Pörtner et al. (29) saw concomitant increases in oxygen consumption and blood lactate concentration in toads. Hypoxic toads injected with a pH-neutral sodium lactate solution had higher metabolic rates than hypoxic controls, and hypoxic anapyrexia was augmented. Adrenergic antagonists attenuated the metabolic response. The authors (29) postulated that lactate acts as an alarm signal that elicits catecholamine release.

Catecholaminergic neurons synapse in the telencephalon (26). In goldfish, central catecholamine microinjections induce anapyrexia (35–37). Catecholamine release by chromaffin tissues of fishes initiates widespread effects on organ systems subserving energy production, oxygen uptake and transport, and osmoregulation. If lactate activates adrenergic systems, the duration of anapyrexia seen in experiment 4 may reflect the time required for catecholamine elimination from blood and thus for removal of its stimulation of central nervous system and/or chromaffin tissues. The time required for catecholamine degradation following removal of lactate or another releaser is another possible explanation for the response duration.

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REFERENCES