低温空気条件におけるコイの鰓機能

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Gill Function of Common Carp in Cold Air Conditions

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To define the role of the gill under cold air, common carp *Cyprinus carpio* acclimated to 17°C were exposed to 7°C air after being equipped with a hood, mask, plug, or bag, and their survival percentage was obtained. Secondly, carp acclimated to 7°C were exposed to 7°C air for 6.5–8 h, 1) to analyze blood pH and gas, 2) to observe the gill histologically, and 3) to measure the activity of succinate dehydrogenase (SDHase) by methylene blue reduction for tissues including the gill of the specimens.

The survival percentage suggested that respiration via the gill occurred to a certain extent in cold air. This was supported by the results of blood gas analysis, histology, and SDHase measurement; in survivors, P_{O_2} showed an increasing tendency, and secondary lamellae as well as SDHase activity of the gill were revealed to be in an active condition.

In the transportation of live fish under cold air conditions, complicated mechanisms or factors may be involved in maintaining the endurance of the live animal. The fundamental mechanism would relate to the energy metabolism and respiration of fish in a cold air environment, and this subject involves immediate adaptation[1] or adjustment.

However, there has been no physiological or biochemical study related to fish under cold air conditions, except a trial experiment with the crucian carp *Carassius carassius cuvieri*[2] and the author's previous report with the common carp *Cyprinus carpio*.[3] The physiological mechanism of the above endurance needs to be proved by indicating the existence of a respiratory function in cold air. The biochemical mechanism needs to be substantiated by proving enzymatic adaptation or measuring enzymatic activities involved in the energy producing pathways.

The present study was conducted with the common carp firstly to define the gill importance for cold air tolerance by an equipped experiment, secondly to prove the respiratory function during cold air life by analyzing blood gas, and thirdly to substantiate gill activity by microscopical observation as well as measuring the activity of succinate dehydrogenase (SDHase) of the carp tolerated in cold air.

Materials and Methods

As materials, about 90 young common carp were used in two different experiments. They were purchased from Kagoshima Prefectural Branch of Freshwater Culture. They had been bred in 27–28°C hot spring water for over two months. Before the first experiment, they were acclimated to 17°C for three months in a 200 *l* round tank provided with an aeration pipe centrally situated and a sand filter at the bottom, and were fed with carp pellets (0.4–1% of body weight per day). After the first experiment, the survivors were acclimated to 7°C for three months for the second experiment. They were not fed during the second acclimation period. The water temperature was controlled with cooling equipment (Taitec Co., CL150). Another tank with a similar shape and volume to the above stock tank was placed beside. This tank was a receptacle containing about 80 *l* waste water brought in from the stock tank every day for the first acclimation and every 10 days for the second acclimation via thin vinyl spihon-tubes at the water exchange. Instead of the outflow, about 80 *l* tap water entered into the stock tank, whose inflow and outflow of 25–30 *l* per hour were adjusted to maintain the 17°C or 7°C temperature and the volume of the stock water.

Equipped Experiment with Hood, Mask, Plug, or Bag

The purpose of this first experiment was to define the important role of the gill in cold air conditions, although the organ has not been proved to function as a respiratory site in air as it does in water.

The specimens were allotted for four different

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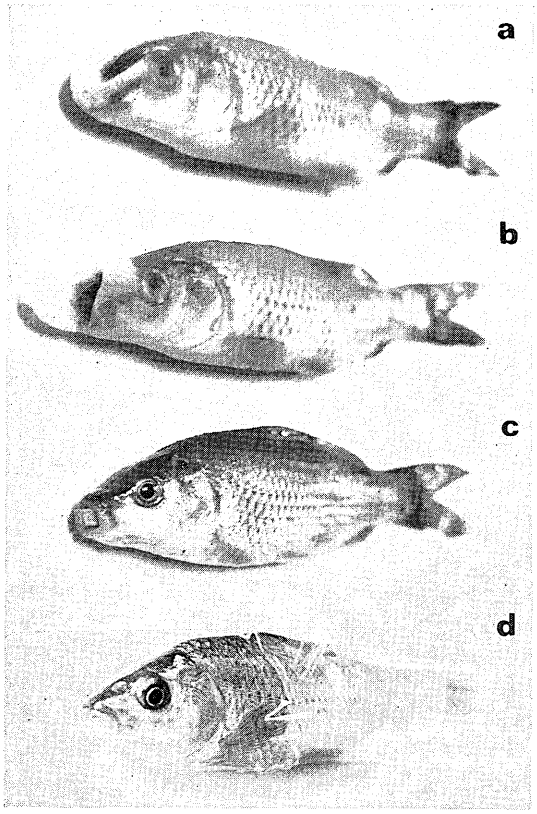

Fig. 1. Specimens in the equipped experiment. Body length is about 9 cm. a) hooded with a fingerstall, b) masked with a fingerstall whose tip is opened, c) plugged with a wooden pile (for demonstration of the plug head, the mouth of carp is opened intentionally), and d) bagged body posterior to the operculum.

treatments: 1) hood over the head and anterior body of the carp, 2) mask over the head and anterior body of the carp, 3) plug inserted into the oesophagus, and 4) bag covering the body posterior to the operculum. The hood, mask, and bag were applied with fingerstalls cut from surgical globes of thin rubber. Their length was 4–7 cm, which was the proper size for the young carp (Fig. 1). In some cases, the rear parts of the hood and mask were held by binder clips in order to be kept in a tight condition. For the bag, its edges were wrapped with a poly-vinylidene chloride sheet. For the mask, the apical stall was cut to expose only the mouth of the carp. For the plug, the treatment aimed to inhibit interference of the air bladder in respiration via the oesophagus. The plug was a conical wooden pile whose head was rectangular, so as to be held easily by tweezers. Even when this was inserted, the carp was still able to move its mouth.

The equipping time was 3.5 h after starting the experiment, *i.e.* precooling with water of a gradually decreasing temperature from 17°C to 7°C for 3 h and 7°C air exposure for 0.5 h. The reason for the 7°C temperature setting was already mentioned previously.[3] The cold air exposure after equipping was continued for up to 6.5 h. Thus the total time for the experiment was 10 h. Cooling conditions were yielded by an incubator of 160 *l* (Hirayama Co., LS611). For each experiment, five carp were initially accommodated in a 7 *l* or 12 *l* acrylic tank which contained water of an adjusted volume similar to a previous study.[3]

At 5, 6, 7, 8, 9, and 10 h after starting the experiment, the survival percentage was obtained from five specimens in each case.

To distinguish surviving and dead carp, recovery tests were conducted successively after air exposure. The carp released from the equipment were returned to 18–19°C water in a basin, and their behaviour was observed for up to 5 min. The waste water provided by the water exchange was applied to this recovery medium. The carp were regarded as dead when they did not show any signs of life such as swimming or movement of the fin or operculum.

The above four experiments together with the control were repeated three times each. The survival percentages for the three values were averaged, and a characteristic curve of endurance according to the lapse of time was obtained for each equipped experiment.

During the experiment, repeated use of the same individual was avoided by marking the tail fin with a punch-mark.

Blood Gas Analysis

As the first part of the second experiment, a hematological approach was conducted to obtain a corroboration of the respiratory function in cold air exposed carp. Carp acclimated to 7°C were exposed to 7°C air without precooling. They were sampled and weighed at 6.5–8 h after air exposure. Surviving and dead specimens as well as control carp in the stock tank were subjected to blood gas analysis. Their body weight ranged from 11 g to 18 g.

Blood sampling followed the tail-cutting method. Flowing blood was sucked into a heparinized 100 μl glass capillary (Ciba-Corning, No. 471819). The blood pH, P_{CO_2}, and P_{O_2} were measured for

each carp with a pH/blood gas analyzer (Ciba-Corning, No. 238) at 37°C calibration to distinguish small differences in the partial pressure of gas between measured values.

Histological Preparation of Gill Lamellae

As the second part of the second experiment, gills were sampled from each of several specimens belonging to the control, surviving, or dead carp in the preceding part. They were fixed in Bouin solution overnight, then immersed in 5% trichloroacetic acid solution to decalcify the tissue for one day. After dehydration by ethanol series, they were embedded in paraffin. Slices of about 1–2 μm were obtained by cutting with a microtome. They were stained with hematoxylin-eosin.

Measurement of SDHase Activity in Brain, Gill and Muscle

As the third part of the second experiment, the specimens supplied for blood sampling in the preceding part were used after stocking for two weeks in a −35°C freezer. To investigate the enzymatic activity related to the TCA cycle, SDHase was applied to this experiment. The brain, gill, and muscle of control, surviving, and dead carp were dissected as quickly as possible during the thawing process. For the brain, all portions of 0.14–0.18 g were extirpated from the skull. For the gill, tissue of 0.35–0.60 g contained gill lamellae and gill arches. The muscle of 0.6–1.1 g corresponded to epaxonal and hypaxonal muscles of the anterior caudal region. They were homogenized individually with an ice-cooled mortar and pestle, with added quartz sand and a saline solution buffered with phosphate (pH 7.6) (Whittaker, No. 17–516B). The added volume of the solution was 10 times the tissue weight for the brain and gill, or 5 times the tissue weight for the muscle. One m*l* of each homogenate was transferred into the accessory receptacle of a Thunberg tube. The main tube contained 2 m*l* of 0.1 M HEPES buffer (pH 7.6) (Dojin, No. 346–01373) and one m*l* each of 0.02 M sodium succinate and methylene blue solution at 0.001% for the brain and gill, or 0.005% for the muscle. After air-aspirating the tube with a vacuum pump (Sato, No. PST-20; exhaust rate 50 *l*/min) for 2 min, solutions of the homogenate and substrate were mixed, and the Thunberg tube was warmed to 30°C. Then the reduction rate of SDHase was measured as the decoloration time (min) of methylene blue[4]; the reciprocal of the time required reflects a temporary SDHase activity applicable only to the same tissue in different individuals. To examine the inhibitory effect of malonate on SDHase, sodium malonate was added to the substrate solution to bring it up to a 0.02 M solution, and the decoloration time of methylene blue was measured.

Results

Equipping Effect on Survival Percentage

In the present study, the experimental carp did not show such violent behaviour during precooling as in the previous case,[3] while struggling and flapping were observed only during the subsequent period of early air exposure.

The following period of air exposure caused the carp to lie down, exhibiting intermittent respiratory movements such as opening and shutting the mouth and operculum in the control, plug, and bag individuals. Such external symptoms of respiratory functions disappeared with longer air exposure. Air exposure of 4–6.5 h dried the surface of the carp body.

Rigor mortis was not recognized in all dead individuals, contrary to the previous case.[3]

The recovery of experimental fish to normal behaviour was very rapid, and the distinction between life and death was always possible within 5 min, contrary to the previous case in which a longer time of up to 50 min in some cases was required by carp acclimated to 27°C.[3]

The hooded specimens showed a decreasing tendency in survival percentage according to the air exposure time (Fig. 2). For 2.5 h after being equipped (corresponding to 6 h in the diagram), the value was 100%. Then, their mean±S.D. became 80±16.3% and 66.7±9.4% one and two hours later, respectively. At 6.5 h after being equipped (corresponding to 10 h in the diagram), the value decreased to 0%.

Weak hyperemia or hemorrhage was infrequently observed on the superficial abdomen of the sampled specimens. Some of the survivors died within a few days in the stock tank.

For the masked equipment, the survival curve showed a similar tendency in percentage change to the hooded specimens according to the time lapse of air exposure (Fig. 2). However, its rate of decrease was faster than that of the hood. For 2.5 h after being equipped, the value was 100%, but after this its mean±S.D. became 60±32.7%

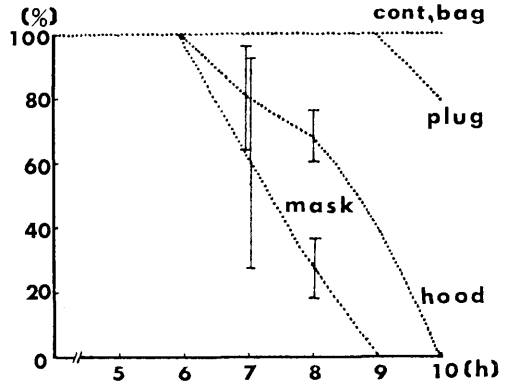

Fig. 2. Characteristic curves of the mean survival percentage after the respective equipping treatment with the hood, mask, plug, or bag.

The control value is added. The vertical line indicates the standard deviation at respective mean values. Abscissa, time after starting the 3 h precooling with water; Ordinate, survival percentage. Experimental period, Nov. 14–Nov. 23. Mean body weight± S.D., hood, 13.6±2.2 g; mask, 14.8±2.5 g; plug, 14.9±2.0 g; bag, 14.2±3.0 g; control, 14.2±2.7 g.

and 27±9.4% one and two hours later, respectively. At 5.5 h after being equipped (corresponding to 9 h in the diagram), the value became 0%. In the masked specimens sampled, hyperemia was frequently observed around the anus or along the ray of the tail fin.

For the plug equipment, the 100% survival value was maintained until 5.5 h after being equipped (corresponding to 9 h in the diagram in Fig. 2). The percentage then decreased to 80% one hour later.

For the bag equipment, the 100% survival value was maintained from the time of equipping to the final sampling time. This result in the case of the bag was the same as that of the control (Fig. 2).

Blood pH, P_{CO_2} and P_{O_2} in Cold Air Exposed Carp

Data from the blood analysis were as follows: the mean±S.D. of pH for the control was 7.46± 0.05, being significantly higher than that of the survivors (6.88±0.11; *t*-test, $p<0.0001$) or dead fish (6.64±0.06; *t*-test, $p<0.0001$). For P_{CO_2}, the mean±S.D. (mmHg) were 12.7±2.0, 29.9±8.2, and 37.1±7.5 for the control, surviving, and dead fish, respectively. The differences between the control and survivors and between the control and dead fish were both significant (*t*-test, $p<$ 0.0005 or $p<0.0001$, respectively). For P_{O_2} the mean±S.D. (mmHg) were 26.7±3.4, 31.9±8.6, and 9.5±3.5 for the control, survivors, and dead fish, respectively. The control and survivor P_{O_2} were significantly higher than that of the dead

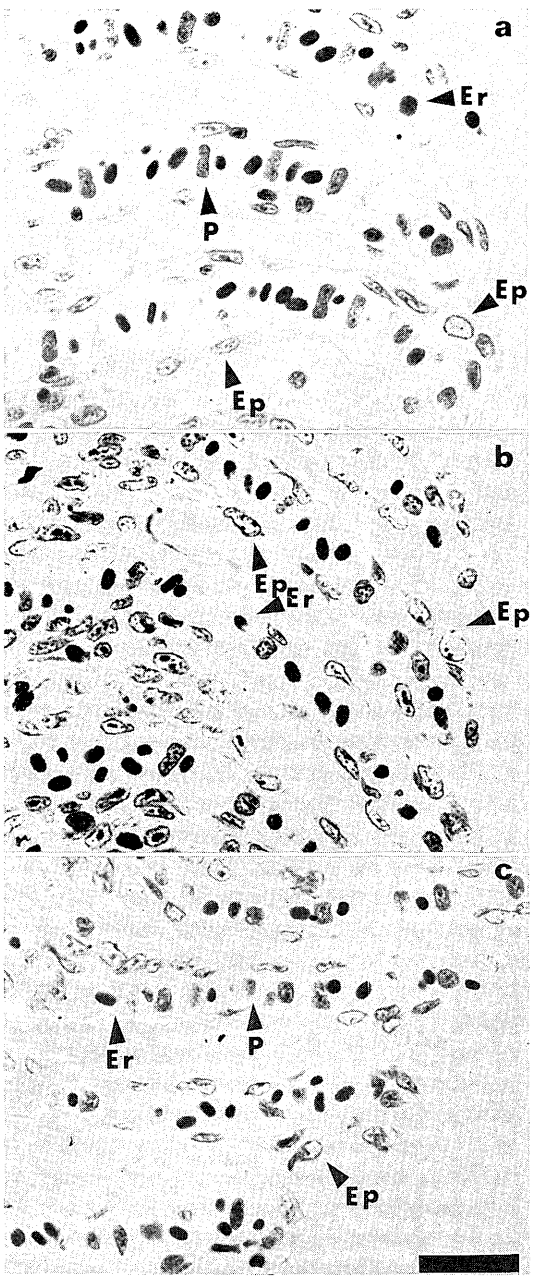

Fig. 3. Micrographs of gill lamellae of control, surviving, and dead carp in the second experiment of 7°C air exposure.

scale bar=20 μm. a) control, b) survivor, c) dead. Abbreviations: Ep, epithelial cell; Er, erythrocyte; P, pillar cell.

Table 1. SDHase activity in the brain, gill, and muscle of carp exposed to 7°C air, measured by reduction time (minutes) of methylene blue at 30°C

tissue	Control (*n*)*3	Survivor (*n*)*3	Dead (*n*)*3
brain*1	6.6±1.17 (10)	6.1±1.14 (10)	6.8± 1.06 (9)
gill*1	49.4±7.76 (8)	38.0±5.87 (10)	51.7±10.33 (6)
muscle*2	5.4±1.20 (10)	5.6±1.48 (10)	6.0± 1.66 (10)

*1 homogenized with a 10 times volume solution to the tissue weight.
*2 homogenized with a 5 times volume solution to the tissue weight.
*3 mean±S.D. (sample number).

fish (each *t*-test, $p<0.0001$). However, the difference in P_{O_2} between the control and survivors was not significant, though an increasing tendency of the mean value of P_{O_2} was evident in the surviving fish.

Observation of Gill lamellae in Cold Air Exposed Carp

Gill lamellae of the survivors showed an active condition in epithelial cells; their nuclei were expanded, and their chromatin was dispersed (Fig. 3b). No such condition was recognized in the control specimens (Fig. 3a). However, the arrangement of the pillar cells was irregular compared to that of the control. For the dead specimens, the active condition of the epithelium seemed to remain (Fig. 3c). The pillar cells also showed a similar situation to that of the survivors. In addition, the thickness of the gill lamellae of the dead fish seemed to be lean, and their epithelium had cellular fragments protruding externally. These results indicate the occurrence of structural shrinkage and damage to the respiratory functions in the gills of dead specimens.

SDHase Activity of Brain, Gill, and Muscle in Cold Air Exposed Carp

The measured value of the time required for decoloration of methylene blue is shown in Table 1. Of three kinds of tissues, significant differences in the mean value were only present in the gill; the lowest value of the survivor gill was significant compared to that of the control (*t*-test, $p<0.005$), or that of the dead fish (*t*-test, $p<0.01$). This indicates that surviving carp had a higher activity of SDHase in the gill than the control and dead fish. For the brain, the SDHase of the survivors also showed the lowest value compared to that of the control and dead fish, though these differences in values were statistically insignificant. Malonate showed an inhibitory effect on the above decoloration reaction.

Discussion

In the present study, the carp transferred to cold air struggled and flapped initially, and intermittent respiratory movements of their mouth and operculum continued to a certain later period. This observation seems to have indicated that the specimens needed proportionately more oxygen for exercise in cold air conditions. This indication agrees with the result of the equipped experiment; only the plug and bag treatments could maintain the high endurance of the specimens to air conditions, and the reason for this seems to be that this equipment gave the specimens both movable mouth and operculum, which were necessary physically in order to maintain respiratory functions.

Therefore, the gill seems to have worked to a certain extent as an important site of respiration even in such air conditions. Another possibility, namely the participation of skin respiration[5] in the above situation, is refuted by the results of the hood and bag equipped experiments, whether they worked to some degree or not under such cold and air conditions. This is because the hooded fish showed a short survival time. The bagged fish showed an equivalent endurance to the control. For the crucian carp *Carassius carassius cuvieri*, the respiratory function of the gill has also been suggested to be important for 20 h survival in 9.5–13.5°C air, as seen in the result of the experiment of operculum cutting.[2]

This assumption was supported by the result of the blood gas analysis; the surviving carp after 6.5–8 h in 7°C air showed a higher tendency in their P_{O_2} value than that of the control, although the variance was large. Taking into consideration the significantly high P_{CO_2} of the survivors, carp in air seem to have respired abnormally without complete gas-exchange; namely, carbon dioxide seems to have remained in the blood without being exhaled. The high P_{CO_2} of the surviving or dead fish may relate to a strong

acidosis shown in respective specimens. As concerns this acidosis, its pH compensation as well as acid-base regulation has been examined in fish, including carp.[6] However, it is difficult to explain precisely the acidosis of the present study according to the result of previous studies. This is because all the previous experiments were conducted for fish not in air but in water.

The 20°C values of pH, P_{O_2}, and P_{CO_2} calculated from the control mean of 37°C by temperature-correction formulae of the blood-gas analyzer are 7.72, 22 mmHg, and 6 mmHg, respectively. Despite such a correction, which allows the values to approach those measured at temperatures studied previously,[6,7] they show differences against the latter obtained from 0.5–0.6 kg or 1.78 kg carp; the latter are 7.89, 24.8 mmHg, and 3.87 mmHg at 24.5°C,[7] or 7.87, 44.4 mmHg, and 4.75 mmHg at 15°C,[6] respectively. These differences between the calculated values in the present study and the previous data are thought to be attributable to differences in fish activity related to body size, rearing conditions, and especially blood samples supplied from the artery via a cannula or collected by the tail-cutting method.

Another result supports the above assumption of the presence of functional gills during cold air life. Of the values of SDHase activity in the control, surviving, and dead fish, the survivors showed the shortest time of methylene blue reduction. This enzyme works as one component of the TCA cycle which produces energy and is essential to an aerobic metabolism. Further, the experimental result of malonate, which is known to be a competitive inhibitor, confirmed the above reaction to be attributable to SDHase activity. Therefore, the present result indicates that the gill's aerobic metabolism even in carp exposed to 7°C air lasted for 6.5–8 h.

Prolongation of the survival time of carp in cold air was yielded by decreasing the acclimation temperature to 17°C in the control of the present experiment; in comparision with the value of 2 h as a net 100% survival time for 27°C acclimation in the previous study,[3] the present 17°C acclimation caused a net 100% survival time of 6.5 h. Further, 7°C acclimation seemed able to yield almost an equivalent survival time despite non-precooling to that of 17°C acclimation with 3 h precooling.

However, this prolongation does not seem only to be due to physiological causes such as low energy consumption of the basal metabolism as well as low respiratory function under such a cold temperature, although the respiratory rate of 17°C or 7°C was reported to decrease to 45% or 12% of that of 27°C.[8] As for the possibility of participation of an anaerobic metabolism[9] in this prolongation, it seems negative. This is because if this metabolism had been functional even in 7°C air as in cold water,[9] such a prolongation as well as the short life in hooded carp would not have occurred.

The histological observation of gill lamellae indicated a structural endurance in the tissue; only dead carp received considerable damage to the epithelium and pillar cells.

Based on the above-mentioned results and consideration, the endurance of carp of cold air in terms of immediate adaptation seems to depend physiologically on the respiratory function of the gill, and morphologically on its structural endurance.

Hyperemia or hemorrhage recognized in the hooded and masked specimen would have occurred due to long abdominal tightening by each rubber stall. For rigor mortis, its non-occurrence in the present study, differing from the previous result,[3] seems to result from the adaptation of enzymes to the low temperature during the previous acclimation.[10]

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