Environmental factors affecting the quality and quantity of hemoglobin in *Chironomus* larvae (Diptera: Chironomidae)

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(Received: 6 February 2004; Accepted: 20 July 2004)

Abstract: The total Hb content and the electrophoretically distinguishable components were compared among 10 species of *Chironomus*. The respiration rates and the survival rates under anoxia were also evaluated for all species. Although larvae used for experiments were all reared under the same laboratory condition, the Hb content and composition differed interspecifically, suggesting genetic control of Hb production in *Chironomus*. However, the phylogenetic relationship inferred from a cluster analysis of Hb components differed greatly from the phylogeny based on randomly amplified polymorphic DNA (RAPD) analysis. The phylogenetic congruence suggests that some convergent evolution occurs in Hb composition of some lentic *Chironomus* species. Among the three factors, respiration rate, total Hb content and Hb diversity, the survival duration under anoxia was significantly correlated with the Hb content and respiration rate. In the species inhabiting a lentic environment where the chironomids are frequently exposed to low oxygen conditions, high respiration rates and high survival rates may be achieved by possession of abundant and specific Hbs compared with the lotic species' Hb.

Key words: anoxic survival, *Chironomus*, hemoglobin, respiration rate

INTRODUCTION

Chironomid larvae of the genus *Chironomus* are known to live in a wide variety of waters; e.g., eutrophic lake, polluted river, brackish water, hot springs and acidic water (Sasa and Kikuchi, 1995). Interspecific differences in habitats are owing to their preference for and/or tolerance to various environmental conditions. Larvae of most species of this genus possess a large amount of hemoglobin (Hb) in the body fluid, comprising many components which differ interspecifically (Baur et al., 1983; Kawai and Sakamoto, 1992).

To clarify how environmental factors contribute to various respiratory properties, the total Hb content and the elec-

trophoretically distinguishable Hb components as well as survival rates under anoxia and respiration rates were compared among 10 species of *Chironomus*. The determinant factors for Hb composition and concentration were discussed.

MATERIALS AND METHODS

Chironomids and rearing method

Mature larvae of all species except for *Chironomus plumosus* (Linnaeus, 1758) were obtained by laboratory cultures. Adult midges of *C. fusciceps* Yamamoto, 1990 were stenogamic, and fertilized egg masses were easily obtained in a small space (5 cm in height). As for *C. kiiensis* Tokunaga, 1936, *C. okinawanus* Hasegawa et Sasa, 1987 and *C. javanus* (Kieffer,

1924), a medium space (30 cm at the highest) was required for fertilization. For the remaining 5 species, *C. circumdatus* (Kieffer, 1916), *C. flaviplumus* (Tokunaga, 1940), *C. nippodorsalis* (Sasa, 1979), *C. nipponensis* Tokunaga, 1940 and *C. yoshimatsui* Martin et Sublette, 1972, fertilized egg masses were obtained in a large space (higher than 30 cm). Fertilized egg masses of *C. plumosus* could not be obtained in the laboratory, and thus mature larvae were obtained by rearing the hatchlings of the egg masses laid by females captured in the field. Species identification of *C. plumosus* was based on the emerged male adults according to the taxonomic key by Sasa and Kikuchi (1995).

For rearing experiments, a milk-containing agar medium, originally described in Shirota (1969), was used as follows. Newly hatched larvae were reared at a density of 100 individuals per plastic container ($\phi 15 \times H9$ cm) at the room temperature. Each container was layered with 200 ml of 1% (w/v) agar containing 2% (v/v) milk (fat content 3.5%) at the bottom, filled with 600 ml of dechlorinated water and aerated through an airstone. As the material for tube (or case) building, 50 ml of glass beads (0.1 mm in diameter) was spread on the agar plate.

Hb analyses

Larvae are usually very small and difficult to weigh individually, thus individual quantification of Hb was practically impossible. Therefore, an adequate number of larvae was used as a sample and measured for wet weight, and total Hb was prepared by homogenization of larvae in a small amount of distilled water, followed by centrifugation of the homogenate at 13,000 rpm for 10 min at 4°C and lyophilization of the supernatant.

Total Hb content ($\mu\text{g}/\text{ind.}$) was measured by the cyanmethemoglobin method (van Assendelft and Zijlstra, 1975) using Nescort hemokit-N (Nihon Shoji Co., Ltd., Osaka, Japan) as a standard solution.

Hb components were separated by poly-

acrylamide gel electrophoresis (PAGE), on the basis of Laemmli (1970), with some modifications. The total Hb was mixed with a sample buffer, pH 6.8, without 2-mercaptoethanol, and electrophoresed at 30 mA for 2 hrs. The gel plate was composed of a stacking gel (4% acrylamide in 0.125 M Tris-Cl, pH 6.8) and a separation gel (15% acrylamide in 0.375 M Tris-Cl, pH 8.8). After running, the gel was stained with a DAB buffer (Merck, Darmstadt, Germany) using benzidin reaction.

Template DNA preparation and RAPD analysis

Template DNA for PCR was prepared using Easy-DNA™ Kit (Invitrogen, San Diego, CA, USA), according to the manufacturer's instruction. Randomly amplified polymorphic DNA (RAPD) analysis was performed using a kit (Ready-To-Go RAPD Analysis Beads, Amersham Biosciences, Piscataway, NJ, USA) with a thermal cycler (THERMO PROCESSOR TR-100, Taitec, Koshigaya, Saitama, Japan). One (Primer 04) of a set of oligonucleotides (RAPD Analysis Primer Set, Amersham Biosciences, Piscataway, NJ, USA) was used as a PCR primer. The sequence was 5'-AAGAGCCCGT-3'.

Calculation of dissimilarity (D) value

D value (Gilbert et al., 1990) was calculated by $D = N_{ab} / (N_a + N_b)$, where N_{ab} was the number of fragments which were not shared by individuals a and b, and N_a and N_b were the number of fragments for individuals a and b, respectively. A dendrogram was constructed by the UPG (Unweighted Pair-Group Clustering) method (Nei, 1975).

Survival duration in an anoxic condition and respiration rates

Survival in an anoxic condition was examined by the method described by Hirabayashi and Hayashi (1994). In brief, five to 15 individuals of the 4th instar larvae of each species were put into the BOD bottle (Sibata Scientific Technology Ltd., Tokyo,

Japan) in which dissolved oxygen had been almost completely driven out (<0.5 mgO₂/l) by bubbling of nitrogen air. Survival was observed at 6-hour intervals until all the individuals died. Death was judged as a lack of undulational behaviour, no response to physical stimuli, and/or changes in body shape and color. Time for 50% survival was estimated on the basis of survival curves. The experiments were performed 4–8 times for each species.

Respiration rate was measured with a BOD bottle of approximately 100 ml in volume. Ten to 50 individuals of the 4th instar larvae were put into the bottle at 25°C which was filled with water saturated with oxygen by air bubbling and spread with 10 ml of glass beads. A bottle without larvae was also incubated at 25°C as a control. Three hours later, dissolved oxygen concentration in the water was measured by Winkler's method (Alsterburg, 1925). Respiration rate ($\mu\text{gO}_2/\text{hr}/\text{indiv.}$) was calculated from the difference in the amount of dissolved oxygen between the control bottle and the sample bottle. The experiments were performed 7–12 times for each species.

RESULTS

Hb content and components

Hb content was higher than 150 μg for 3 large species, *C. nippodorsalis*, *C. nipponensis* and *C. plumosus*. It was low (<40 μg) for *C. fusciceps*, *C. javanus* and *C. yoshimatsui* and was in the range of 40–70 μg for other species (Table 1). Differences in Hb components among the species are shown in Fig. 1. A total of 25 different Hb bands was detected by PAGE analysis and they were numbered in the order of the distance from the electrophoretic origin. Some bands were common to different species. The band 25 was common to all species except for *C. fusciceps* and *C. okinawanus*. The bands 1 and 13 were common to *C. fusciceps*, *C. kiiensis*, *C. flaviplumus*, *C. yoshimatsui*, *C. circumdatus* and *C. nipponensis*; and *C. fusciceps*, *C. flaviplumus*, *C. javanus*, *C. okinawanus*, *C. nippodorsalis* and *C. nipponensis*, respectively. The bands 7 and 11 were common to *C. fusciceps*, *C. flaviplumus*, *C. javanus*, *C. circumdatus* and *C. plumosus*; and *C. flaviplumus*, *C. okinawanus*, *C. nippodorsalis*, *C. nipponensis* and *C. plumosus*, respectively. On the other hand, some bands were species-specific. The bands 4; 14 and 20; 18; 21; 22; 23; and 24 were specific to *C. fusciceps*;

Table 1. Body wet-weight, 50% survival duration in anoxic condition, respiration rate, Hb content and Hb composition of 10 *Chironomus* species.

Species	Body wet-weight (mg)	Survival duration (hr)	Respiration rate ($\mu\text{gO}_2/\text{hr}$)	Hb content (μg)	Hb composition (no. of bands)
<i>C. fusciceps</i>	1.96	10.0 $\pm$ 1.15 ^a (7)	1.08 $\pm$ 0.25 ^a (9)	37.5	5
<i>C. kiiensis</i>	2.23	27.3 $\pm$ 3.13 ^c (6)	1.78 $\pm$ 0.38 ^{cd} (9)	61.9	7
<i>C. flaviplumus</i>	2.68	23.8 $\pm$ 3.19 ^b (6)	1.85 $\pm$ 0.31 ^{cd} (9)	69.6	8
<i>C. javanus</i>	2.81	9.93 $\pm$ 2.30 ^a (8)	1.43 $\pm$ 0.17 ^b (8)	39.0	6
<i>C. yoshimatsui</i>	2.86	22.7 $\pm$ 1.54 ^b (6)	1.92 $\pm$ 0.59 ^{cde} (8)	37.5	11
<i>C. okinawanus</i>	2.95	11.2 $\pm$ 0.00 ^a (5)	1.56 $\pm$ 0.40 ^{bc} (9)	44.4	4
<i>C. circumdatus</i>	3.53	27.0 $\pm$ 1.60 ^c (5)	1.55 $\pm$ 0.54 ^{bc} (12)	56.5	6
<i>C. nippodorsalis</i>	4.06	34.4 $\pm$ 4.79 ^d (6)	2.23 $\pm$ 0.55 ^{de} (9)	185	6
<i>C. nipponensis</i>	4.29	27.3 $\pm$ 6.38 ^{bc} (7)	2.45 $\pm$ 0.58 ^e (7)	151	8
<i>C. plumosus</i>	11.3	24.7 $\pm$ 1.06 ^b (4)	3.40 $\pm$ 0.49 ^f (8)	222	7

Mean $\pm$ standard deviation and the number of experiments (in parentheses) were shown for survival duration and respiration rate. Values within columns followed by different superscript letters a–f are significantly different ($P<0.05$) by Student's *t*-test.

Fig. 1. Hb components for 10 species analysed by PAGE. Each Hb band was numbered in the order of the distance from the electrophoretic origin.

Fig. 2. A, A clustering pattern of 10 species on the basis of Hb components; B, A clustering pattern of 10 species on the basis of DNA fragments amplified in RAPD-PCR.

C. yoshimatsui; *C. circumdatus*; *C. javanus*; *C. nipponensis*; *C. okinawanus*; and *C. nipodorsalis*, respectively.

Phylogeny on Hb components and DNA

Clustering on the basis of Hb composition is shown in Fig. 2A. A large cluster was made by all species except for *C. oki-*

Fig. 3. Relationships among some respiratory parameters. A, Relationship between the residual respiration rate and survival duration ($r=0.70$, $P<0.05$); B, Relationship between the residual Hb content and survival duration ($r=0.65$, $P<0.05$); C, Relationship between Hb composition and survival duration ($r=0.41$, $P>0.05$); D, Relationship between the residual Hb content and the residual respiration rate ($r=0.66$, $P<0.05$).

nawanus and *C. yoshimatsui*, which were both distant from the other 8 species. *C. javanus* and *C. plumosus*; and *C. fusciceps* and *C. nipponensis*, each made a small cluster. *C. nippodorsalis*, *C. flaviplumus* and *C. kiiensis* made another small cluster, among which the latter two species made a relatively intimate cluster.

Clustering on the basis of the DNA fragments amplified in RAPD-PCR is shown in Fig. 2B. Largely three clusters were made; *C. fusciceps*, *C. circumdatus*, *C. okinawanus* and *C. yoshimatsui*; *C. nippodorsalis*; *C. flaviplumus*, *C. javanus*, *C. plumosus*, *C. kiiensis* and *C. nipponensis*. *C. okinawanus* and *C. yoshimatsui*; *C. flaviplumus* and *C. javanus*; and *C. kiiensis* and *C. nipponensis*, each constructed a small cluster.

Anoxic tolerance and the factors affecting it

The body wet-weight was by far the highest for the largest species, *C. plumosus* and was in the range of 2.0–4.3 mg for all other species (Table 1). The mean 50% survival duration of *C. nippodorsalis* in

anoxic conditions was significantly the longest (34.4 hr) among the species ($P<0.05$, t -test). Those of *C. fusciceps*, *C. javanus* and *C. okinawanus* were significantly shorter (9.93–11.2 hr) than that of the other species ($P<0.05$, t -test). It was in the range of 22.7–27.3 hr for all other species. The mean respiration rate of *C. plumosus* was significantly the highest among the species ($P<0.05$, t -test). That of *C. fusciceps* was significantly the lowest among the species ($P<0.05$, t -test). It was in the range of 1.43–2.45 $\mu\text{g O}_2$ for all other species.

There were significant positive relationships between body wet-weight and respiration rate ($r=0.89$, $P<0.01$) and between body wet-weight and Hb content ($r=0.81$, $P<0.01$) (Table 1). However, there were no significant relationships between body wet-weight and mean survival duration ($r=0.27$, $P>0.05$) and between body wet-weight and Hb composition ($r=0.06$, $P>0.05$) (Table 1). Besides, there were no significant relationships between Hb com-

position and mean survival duration ($r=0.41$, $P>0.05$) (Fig. 3C). Therefore, the factors affecting the survival duration under anoxia were analyzed using the residuals for respiration rates and Hb contents. As a result, the survival duration was longer with increasing respiration rates ($r=0.70$, $P<0.05$) (Fig. 3A) and Hb contents ($r=0.65$, $P<0.05$) (Fig. 3B). The respiration rates were also correlated with the Hb contents ($r=0.66$, $P<0.05$) (Fig. 3D).

DISCUSSION

In this study, Hb content was high in *C. nipodorsalis*, *C. nipponensis* and *C. plumosus*, all living in lentic environments (Sasa, 1978; Yasuno et al., 1983) whereas it was low in *C. fusciceps*, *C. yoshimatsui* and *C. okinawanus*, all living in lotic waters (Hashimoto, 1977; Sasa and Hasegawa, 1983; Yamamoto, 1990). Besides, the survival duration was longer with increasing respiration rates and Hb contents. The respiration rates were also correlated with the Hb contents. These results suggest that anoxic resistance of lentic *Chironomus* species may be dependent on the Hb content.

On the other hand, *C. yoshimatsui* larvae are found in oxygen-poor sites in a river whereas a non-Hb bearing species, *Cricotopus bicinctus* (Meigen, 1818) larvae are found in more oxygen-rich sites in spite of the same oxygen concentration level at which the two species could compensate the respiration (Ohno, 1984, 1985). This can be explained by the difference in body size between the two species (*C. yoshimatsui* is 4 times larger in body weight than *C. bicinctus*). Thus, a smaller species has a relatively large respiration rate per body weight which is disadvantageous to anoxia (Hayashi, 1989). In this study, however, there was no significant relationship between body wet-weight and survival duration in anoxia. Therefore, the lack of Hb might be partly responsible for the inability of *C. bicinctus* to live in oxygen-poor waters.

In cluster analysis on the basis of Hb

composition, a high level of similarity was observed between *C. flaviplumus* and *C. kiiensis*, both mainly living in stagnant or lentic waters (Hashimoto, 1977). Further, *C. nipodorsalis*, also a lentic species, constructed a cluster with *C. flaviplumus* and *C. kiiensis*. Similarly, *C. javanus*, often emerging from artificial pools, made a cluster with a lentic species, *C. plumosus*. *C. okinawanus* and *C. yoshimatsui* were both distant from the other 8 species and different from each other. These suggest that Hb composition is congruent in lentic environments whereas it is admitted to diverge in lotic environment. In contrast, the clustering patterns were quite different on the basis of RAPD. Namely, the lentic 3 species, *C. flaviplumus*, *C. kiiensis* and *C. nipodorsalis*, each constructed a distinct cluster, and two other lentic species, *C. javanus* and *C. plumosus*, each participated in different clusters. Further, *C. okinawanus* and *C. yoshimatsui* made a cluster. Thus, it may be inferred that Hb composition be determined not by evolutionary factor(s) but by environmental factors of habitats and that there are some evolutionary congruences of Hb composition in the chironomid species.

Although larvae used for experiments in this study were all reared under the same laboratory condition, Hb content and composition differed interspecifically, suggesting some genetic control of Hb production in *Chironomus*. However, for some species of the genus *Polypedilum*, another common genus in the family Chironomidae, Hb composition has been reported to change among different rearing conditions, probably corresponding to differences in water quality, in our previous study (Kawai et al., 2000). Therefore, larval Hb composition of each species, reared in the laboratory, does not always reflect that of living larvae in the fields. It is desirable that rearing methods approximating natural conditions as much as possible be worked out.

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