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Effects of Population Density on Growth, Behavior and Levels of Biogenic Amines in the Cricket, *Gryllus bimaculatus*

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ABSTRACT—We examined quantitative changes in biogenic amines in relation to effects of population density on growth and behavior in the cricket *Gryllus bimaculatus*, which were reared in two different densities, completely isolated solitary and 40 crowded insects. In the crowded animals, the rate of increase in body weight was slow, and also the day of imaginal moult was late, when compared to the isolated ones. Development and growth were thus suppressed in the crowded group. The body color of isolated ones was darker than that of crowded ones. Behavioral tests indicated that the isolated crickets were significantly more aggressive than the crowded ones. The levels of biogenic amines, their precursors and metabolites were examined in the brain, corpora allata, corpora cardiaca and frontal ganglion of isolated and crowded crickets using high-performance liquid chromatography with electrochemical detection. The brains of crowded crickets contained significantly higher amounts of octopamine, dopamine, and 5-hydroxytryptamine (5-HT) than those in the isolated ones. On the contrary, the level of N-acetyldopamine in the isolated crickets was significantly higher than that in the crowded ones. In crowded animals the corpora allata contained significantly higher levels of 5-HT, and the corpora cardiaca contained more octopamine. These results indicate that high population density affects aminergic systems which in turn modulate various biological events, such as development, growth and behavior.

INTRODUCTION

It is well known in many animal species including insects that population density affects development and growth which usually result in morphological changes, and further behavioral patterns. But underlying mechanisms for developmental and behavioral effects are not clear.

The presence of two phases, phase solitaria and phase gregaria, is known in the African migratory locust *Locusta migratoria* and desert locust *Schistocerca gregaria*. In *Locusta migratoria*, both larval and adult colors, the growth rate and the body size are prominent characteristics affected by these phases. Gregarious adult locusts are pinkish brown or bright yellow, whereas solitary adults are green [11]. Morphologically, the gregarious adults have wider heads and longer erytron than the solitary ones [5, 19]. The crowded females required longer time to be matured, and were shorter in longevity than the isolated ones [12].

In pea aphids, *Acyrthosiphan pisum*, an isolated insect is apterous, whereas that reared in crowded is alate. The time required for completion of developmental stages in the apterous is shorter than that in the alate. The apterous insects are heavier than the alate ones, and apterous parents produce a larger number of eggs than alate parents [20]. Density-dependent phase variation also occurs in the migratory insect *Anticarsia gemmatalis*, in which an increase in the population density of larvae facilitates dark pigmentation and prolongs duration of larval stage, however decreases adult body weight [3].

The prominent factor responsible for development and growth is juvenile hormone. Juvenile hormone further has an effect on colorlization. Pener [15] demonstrated that an implantation of corpora allata in young adults of crowded *Schistocerca gregaria* induced very bright yellow, whereas allatectomized animals were pinkish beige. In *Gryllus bimaculatus*, an implantation of adult corpora allata into last instars induced dispersion or disappearance of black pigments, meanwhile allatectomy of last instars induced black pigmentation [18]. In addition, corpora allata and juvenile hormone have effects on oocyte growth in *Rhodnius prolixus* [21] and *Shistocerca gregaria* [15], and male sexual behavior in *Schistocerca gregaria* [15].

Biogenic amines, such as octopamine, dopamine and 5-hydroxytryptamine (5-HT), play important roles in control of various physiological processes in insects as neurotransmitters, neuromodulators and neurohormones [2]. In the cricket *Acheta domesticus*, the 5-HT levels in the brain and hemolymph were varied with the circadian rhythms of locomotor activity [8]. When ants *Formica rufa* displayed

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aggressiveness, the concentrations of 5-HT and adrenaline were elevated in the brain [6]. Goosey and Candy [4] showed that, after 10 min flight, the octopamine level increased about 5 times compared to resting in the locust, *Schistocerca gregaria*. A similar result was also demonstrated in *Locusta migratoria* [1].

In the present study, we examined effects of population density in the cricket *Gryllus bimaculatus* by maintaining them in isolated and crowded conditions. This species, which is widely distributed over Africa to Southern Asia, usually lives solitary in burrows or under stones, diurnal in the larval stage and nocturnal during adult life, although we reared them in a crowded condition in the laboratory. First, the effects of population density were compared on the following points: developmental time for imaginal moult, body weight, male body color and male aggressive behavior. Thereafter, we determined the levels of biogenic amines and metabolites in the brain, corpora allata, corpora cardiaca and frontal ganglion of isolated and crowded insects using high-performance liquid chromatography (HPLC) with electrochemical detection. From the results obtained, we discussed underlying humoral mechanisms for developmental effects of population density.

MATERIALS AND METHODS

Animals

Crickets in breeding colonies were reared in plastic containers of 69 cm × 44 cm × 22 cm at 27–28°C with relative humidity of 45–50% under a 14L : 10D photoperiod, fed on artificial insect diet (Oriental Yeast) and carrot slices. Water was supplied via three cotton wool-plugged glass bottles, which were also used as places for egg laying. The floors of containers were covered with paper towels. Shelters were provided by placing 12 sheets of crumpled paper into each container. Experimental animals were bred from one pair of insects which were separated from the breeding colony just after imaginal moult. They were maintained in a small plastic case (17 cm × 11 cm × 15 cm) for about 10 days, because *Gryllus bimaculatus* mature 3 days after imaginal moult and afterward lay eggs to the cottons for about 7 days. After the female laid eggs, the cottons in which eggs were laid were taken out and spread onto plastic cases. When instars hatched out from the eggs about one week later, they were transferred to another case in which only animals hatched in the same day were included. The methods of rearing was almost the same as described above.

Observation of development

The instars just hatched are so small as 3 mm and may be easily injured if handled, so that they were left untouched in the same case until they shed off their first skins. Thereafter, they were separated into two groups: solitary ones in plastic cases (16.5 cm × 16.5 cm × 9 cm), and crowded ones including 40 individuals in a plastic cases (31 cm × 15.5 cm × 23.5 cm) as the population density became about 800 insects/m^2.

Body weight were measured every other day from day 12 after hatching in isolated crickets and day 22 in crowded crickets. All the isolated crickets were weighed, whereas for the crowded randomly selected 15 insects were measured. The days for the imaginal moult were determined by everyday observation.

Magnitudes of dark pigmentation

The right anterior wing of adult male cricket was cut off 10 days after the imaginal moult, and the tip of the wing was cut about 5 mm square. The sample from the wing tip was then mounted on a slide glass, sealed with Canada balsam, and its digitized monochrome image was acquired into a personal computer through a TV-camera attached to a microscope. The optical density of acquired image was measured for 100×100 pixels with a 256-stage gray scale, assigning black as 0 and white as 255. The gray scale values for individual pixels from 10 wings were accumulated to show the magnitudes of dark pigmentation as distributional histograms.

Observation of aggressive behavior

The aggressive behavior was observed in matured male crickets during the 14L:10D light cycle. The behavior was tested using a plastic chamber (11 cm × 8 cm × 7 cm) with a removable partition which provided two small rooms. Single animals were placed in each of two rooms, and were left separated for 30 min to accommodate a new environment. The partition was then removed, and behavior of 2 crickets was observed for 5 min. The crickets used in the behavioral tests were selected so as to minimize differences of body weights between two animals. The aggressiveness was scored according to the following criteria classified into 7 levels: 0, no response; +1, antennal lashing; +2, standing up; +3, mandible spreading, pushing the opponent backwards and aggressive song stridulation; +4, the same as +3 but more frequently; +5, running after a looser and aggressive song stridulation; +6, a looser injured. The tested pairs were both isolated, both crowded, and the isolated one versus the crowded one.

Levels of biogenic amines

Emerged crickets of crowded were taken out from the colony, and were separated not to mix the newly emerged crickets. Isolated crickets were continuously maintained in the same case they reared after the imaginal moult. Ten days after the imaginal moult, animals were anesthetized with ice and dissected in saline solution consisting of 140 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 44 mM glucose, buffered to pH 7.3 with 2 mM N-Tris (hydroxymenthyl) methyl-2-aminoethanesulfonic acid (TES) (Dotite) and NaOH.

The method of Nagao and Tanimura [9, 10] was applied upon an improvement for determination of the lower levels of biogenic amines at around 10 fmols. Single brains were collected in a microglass homogenizer, while for the others 5 organs were pooled in a microglass and stored at −70°C before use. The samples were homogenized in 40 μl of 0.1 M perchloric acid containing 50 ng/ml 3,4-dihydroxybenzylamine (DHBA) as an internal standard. The homogenate was transferred to a 2.0 ml Eppendorf tube and mixed for 30 min at 4°C. Afterward it was centrifuged at 15,000 G for 30 min at 0°C, and 5 μl of the supernatant was injected to a HPLC column.

The HPLC system was composed of a pump, an injection valve, and a C_{18} reversed-phase column (Shiseido, 4.6 mmø × 250 mm) heated at 30°C in a column oven. An electrochemical detection with a glassy carbon electrode (EICOM, WE-GC) are used. The detector potential was set at 0.88 V versus an Ag/AgCl reference electrode. They were also maintained at 30°C in a column oven. The mobile phase containing 0.18 M chloroacetic acid, 40 μM disodium EDTA was adjusted to pH 3.6 with NaOH. Into this solution, 1.66 mM sodium-1-octanesulfonate as an ion-pair reagent and 8.00% (v/v) CH_3CN as an organic modifier were added. The mobile phase was then filtered with a 0.22 μm filter and degassed. The flow rate was kept at 0.7 ml/min.

The chromatographs were acquired by a computer with a program for chromatography named Maxima (Waters), and after the acquisition, they were processed to obtain the levels of various biogenic amines and metabolites in the same sample by the ratio of the peak height of substances/internal standard (DHBA).

Statistical analysis

Effects of population density on development were statistically analyzed by the Mann-Whitney's *U* test; aggressive behavior, by the Kolmogorov-Smirnov test; and the levels of biogenic amines, by the Student's *t*-test.

RESULTS

Development

The body weight was measured from the day 12 after hatching in isolated crickets and from the day 22 in crowded ones (Fig. 1). On the day 22, there was already a difference between the isolated and the crowded. The body weight of isolated was 0.18 g and that of crowded was 0.11 g. As is clearly shown by the growth curves in Figure 1, the increase in the body weight was much faster in the isolated animals than the crowded. Further, the isolated attained heavier weight than the crowded, when fully grown. Comparison of the body weight on the day of imaginal moult (Fig. 2) yielded a similar conclusion. In the isolated, their body weight

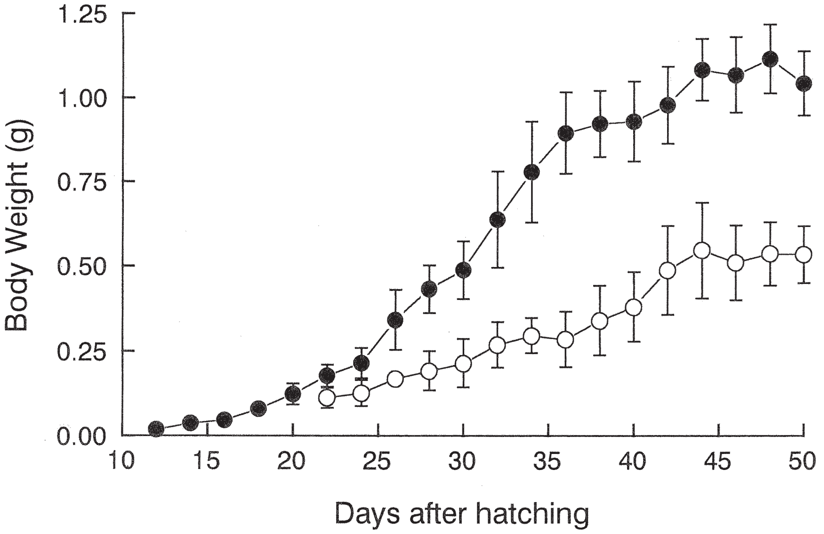

FIG. 1. Effects of population density on increases of body weight measured every other days from the day 12 after hatching in isolated crickets and the day 22 in crowded crickets. Filled circles represent isolated crickets (n=8), and open circles, crowded crickets (n=15), mean ± S.E.

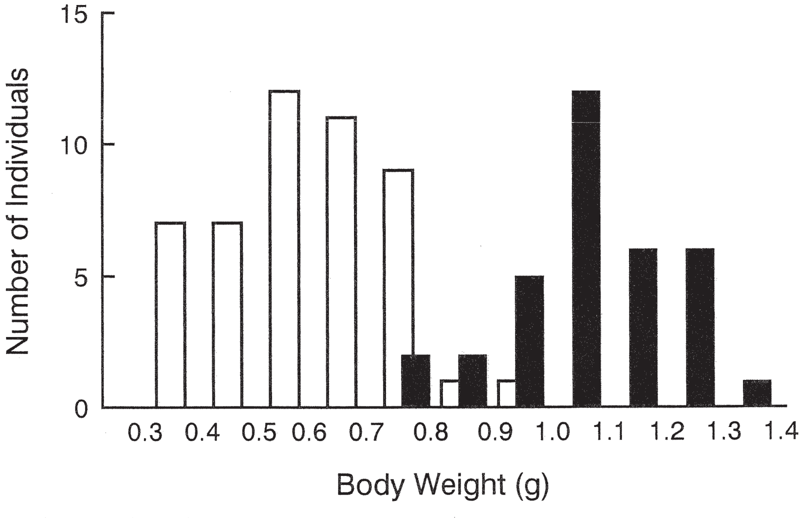

FIG. 2. Distribution of body weight in adult crickets just after the imaginal moult. The range of body weight was from 0.75 g to 1.36 g in isolated crickets and that in crowded ones was from 0.33 g to 0.98 g. Solid bars indicate the number of isolated crickets in the same range (n =34), white bars that of crowded crickets (n=48).

ranged from 0.75 g to 1.36 g, on the other hand, the range of body weight was 0.33 g to 0.98 g in the crowded. The mean body weight of isolated crickets (1.06±0.02 g) was thus twice as heavy as that of crowded ones (0.58±0.02 g) at the time of imaginal moult ($P<0.0001$).

Crowded condition resulted in the delay in initiation of imaginal moult, that was 44±4.0 days in isolated crickets but 56±7.6 days in crowded ones (Fig. 3). In the isolated crickets, adult insects first appeared on the day 38 after hatching and the imaginal moult was observed no later than day 55. In the crowded condition, adult insects first appeared on day 46, 8 days later than the isolated, and the latest instar emerged on day 75 after hatching.

Pigmentation

As is shown in Figure 4, the isolated insects are apparently bigger and darker than the crowded ones. Individual isolated crickets showed several different patterns in dark pigmentation, from almost completely dark to spotted. On the contrary, the crowded was homogeneously brown, and did not show varied patterns. To determine the difference in the magnitudes of dark pigmentation accurately, we measured the darkness of wings by a computer-assisted image analysis, adopting gray scale classified into 256 levels. Histograms shown in Figure 5 A-D are of typical pigmentation patterns of the isolated crickets, and E and F, of the crowded. There was a marked difference in the distributional histograms of gray scale values between the isolated and the crowded (Fig. 5 G, H). The gray scale values of wings from the isolated were widely spread from dark to light without any notable peaks, but those from the crowded were distributed far light with a sharp peak. Thus the body color of the isolated crickets was evidently darker than that of crowded ones. The difference in development and dark pigmentation between the isolated and the crowded may not be derived from hereditary causes but effects of population density, because the pairs of crickets to be parents of experimental animals were randomly selected from the same breeding colonies.

Observation of aggressive behavior

The isolated crickets seemed to have higher activity, and the mature solitary males stridulated more vigorous than the crowded ones. Thus we considered that observation of aggressiveness in mature male crickets can provide good indices to detect effects of population density on behavior. In pairwise trials of both isolated ones, both of encountered crickets initially spread their mandibles and threatened each other for a long time. When one of them won dominance, he ran after the escaping subordinate and sometimes bit looser's legs to injure. On the other hand, in the trials of both crowded ones, such an intense fight shown in both isolated ones were not observed. The fight shown in this case was at the level of aggressive song stridulation by winner

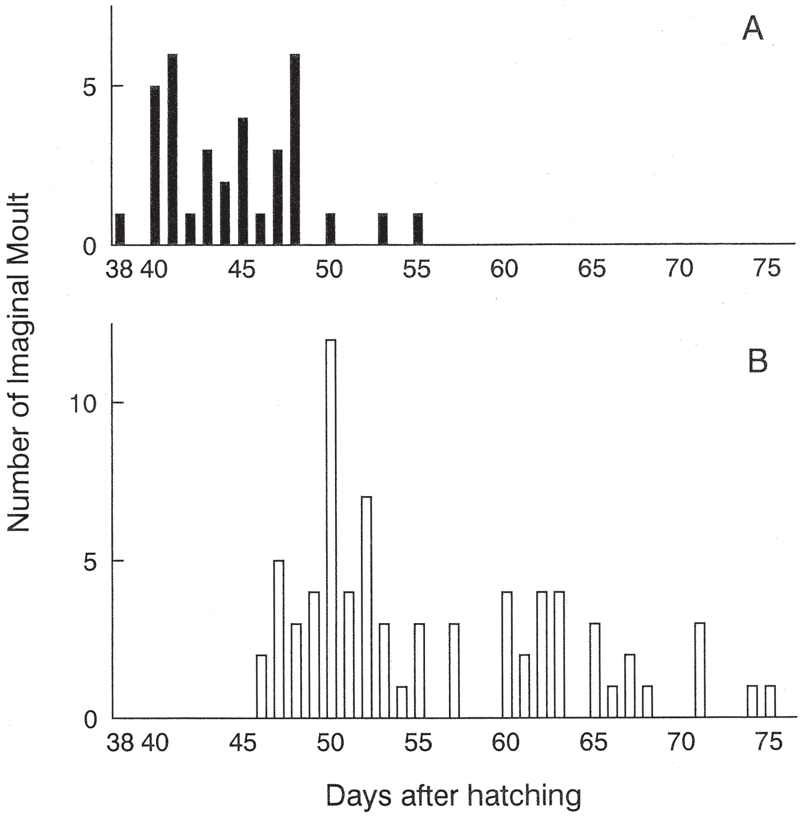

Fig. 3. Effects of population density on the occurrence of imaginal moult. A, isolated crickets (n=35). The numbers of animals of imaginal moult in the same day are shown. B, crowded crickets (n=75).

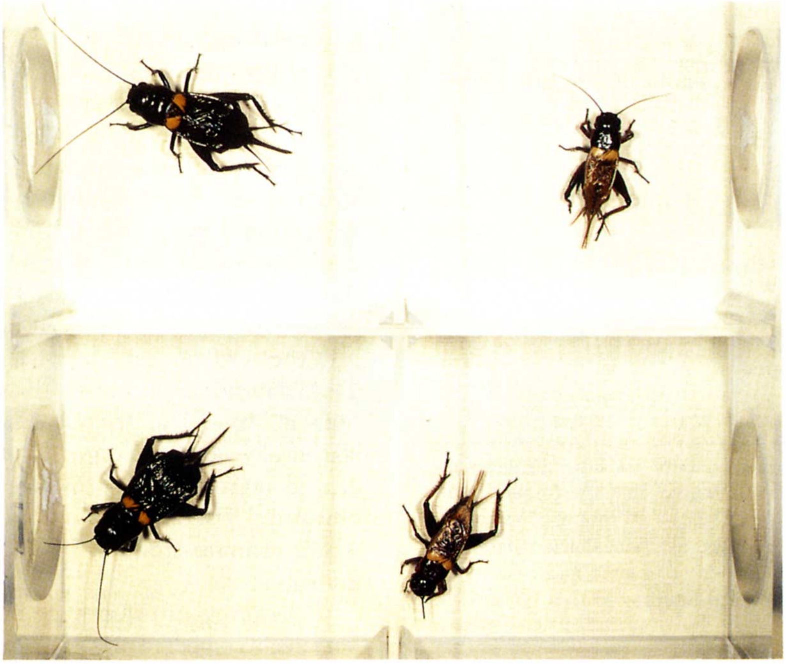

FIG. 4. Photographs of adult male crickets. Crickets shown in the left are isolated crickets and those in the right are crowded ones. Note that the isolated ones are larger and darker than the crowded ones.

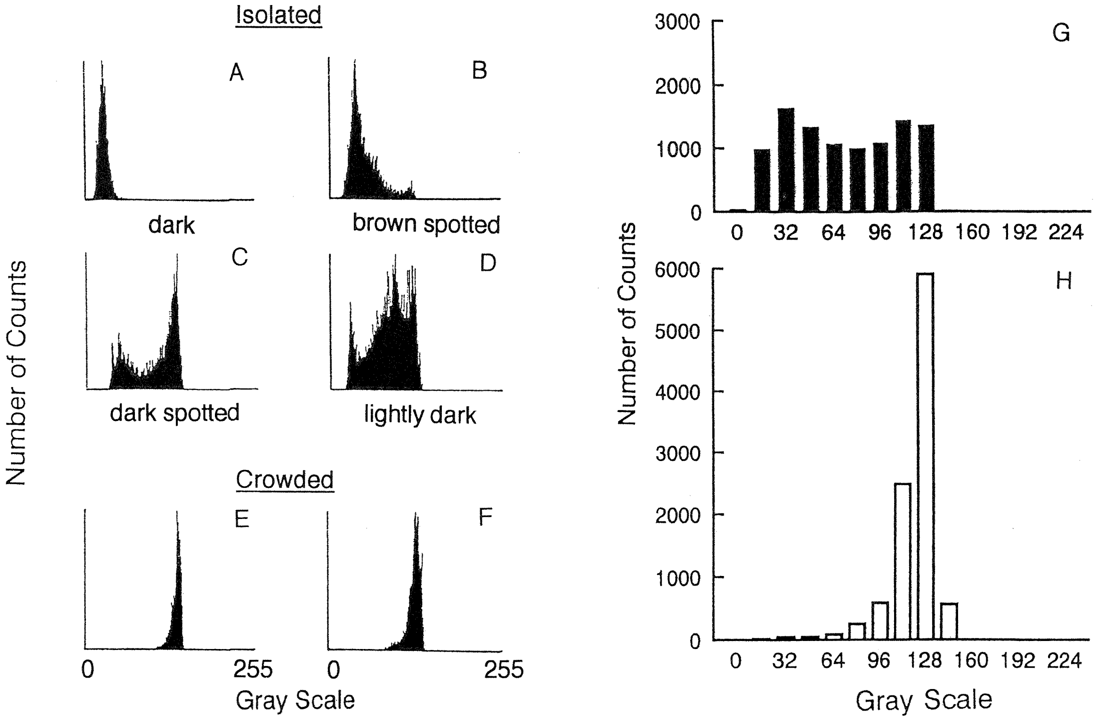

FIG. 5. Effects of population density on body color. A-D, the typical patterns of distribution of dark pigmentation in isolated crickets shown by gray-scale distributional histograms. E and F, in crowded ones. G and H show averaged patterns calculated from the 10 sheets of wings. Solid bars, isolated crickets; open bars, crowded ones.

crickets. In the fight of isolated versus crowded, isolated crickets won at the level as intense as the trials of both isolated ones. However, although only in 2 trials, the significant differences between isolated versus isolated and crowded versus crowded ($P<0.01$), and also crowded versus crowded and isolated versus crowded ($P<0.01$), showing that ones.

TABLE 1. Results of behavioral tests in which aggressiveness was observed for 5 min

Levels of Aggressiveness		Isolated *vs* Isolated	Crowded *vs* Crowded	Isolated *vs* Crowded
0	no response	0	2	0
+1	antennal lashing	0	4	1*
+2	standing up	0	1	0
+3	mandible spreading, pushing other backwards, aggressive song	2	8	1*
+4	same as +3 (frequently)	2	0	2
+5	running after looser, aggressive song	8	0	11
+6	looser injured	3	0	0

Aggressive behavior was classfied into 7 levels, the level of aggressiveness in each trial was scored, and the frequency of each level was obtained after 15 trials. A pair of crowded vs crowded showed rather weak aggressive behavior compared to those observed for pairs of isolated vs isolated and isolated vs crowded ($P<0.01$, by Kolmogorov-Smirnov test). *, crowded one won in the trials.

Levels of biogenic amines

The amounts of biogenic amines and metabolites were determined in the brain, corpora allata, corpora cardiaca and frontal ganglion of both males and females. In these organs, we detected considerable levels of major insect amines, such as octopamine, dopamine and 5-HT, and N-acetyldopamine, N-acetylated metabolites of dopamine. In the brain of both males and females, the levels of octopamine, dopamine and 5-HT were higher in the crowded crickets than in the isolated ones (Fig. 6). The octopamine level in the crowded crickets was twice as large as that in the isolated ones in both sexes. On the contrary, the level of N-acetyldopamine in the isolated was twice as high as that in the crowded, indicating that the brain dopaminergic system was rather suppressed in the crowded compared to the isolated.

The corpora allata of isolated crickets contained about 2.6-fold higher levels of 5-HT than those of crowded animals both in males and females (Tables 2 and 3). Although statistically not significant, the contents of dopamine and N-acetyldopamine were higher in the isolated than the crowded. The differences of octopamine levels between the isolated and the crowded were not sexually consistent in the corpora allata.

The levels of octopamine in the corpora cardiaca was very high compared to the corpora allata and the frontal ganglion (Table 2 and 3). Moreover, in both sexes, the contents of octopamine were significantly higher in the crowded than the isolated. Significant differences in the amounts of dopamine, N-acetyldopamine and 5-HT were not found between the isolated and the crowded.

The frontal ganglion contained a considerable amounts of major insect biogenic amines, however, significant differ-

TABLE 2. The amounts of biogenic amines (pmol) in the corpora allata, corpora cardiaca and frontal ganglion in the male crickets

Biogenic amines	Corpora Allata		Corpora Cardiaca		Frontal Ganglion	
	Isolated (n=12)	Crowded (n=4)	Isolated (n=9)	Crowded (n=4)	Isolated (n=12)	Crowded (n=8)
Octopamine	0.51±0.10	0.29±0.06	1.52±0.30	2.61±0.31*	0.18±0.03	0.25±0.07
Dopamine	0.07±0.02	0.02±0.01	0.15±0.05	0.05±0.03	0.10±0.02	0.15±0.02
N-acetyldopamine	0.36±0.14	0.12±0.01	0.42±0.11	0.26±0.03	0.49±0.09	0.49±0.08
5-HT	0.29±0.09	0.77±0.06*	0.72±0.24	0.99±0.13	0.83±0.32	0.41±0.07

From the values for pooled organs, the amounts in individual pairs of glands and pieces of ganglia were calculated. Then the calculated amounts for the pools were used to obtain the mean±S.E.
*: $P<0.05$, differences between the isolated and the crowded tested by Student's *t*-test.

TABLE 3. The amounts of biogenic amines (pmol) in the corpora allata, corpora cardiaca and frontal ganglion in the female crickets

Biogenic amines	Corpora Allata		Corpora Cardiaca		Frontal Ganglion	
	Isolated (n=3)	Crowded (n=4)	Isolated (n=3)	Crowded (n=4)	Isolated (n=3)	Crowded (n=4)
Octopamine	0.14±0.01	0.22±0.04	0.48±0.15	1.50±0.05*	0.07±0.03	0.21±0.18
Dopamine	0.02±0.003	0.01±0.01	0.04±0.01	0.05±0.01	0.06±0.01	0.08±0.01
N-acetyldopamine	0.15±0.05	0.05±0.01	0.18±0.06	0.23±0.03	0.24±0.002	0.17±0.03
5-HT	0.15±0.07	0.39±0.03*	0.32±0.09	0.40±0.07	0.43±0.22	0.43±0.17

From the values for pooled organs, the amounts in individual pairs of glands and pieces of ganglia were calculated. Then the calculated amounts for 3 or 4 pools were used to obtain the means±S.E.
*: $P<0.05$, differences between the isolated and the crowded tested by Student's *t*-test.

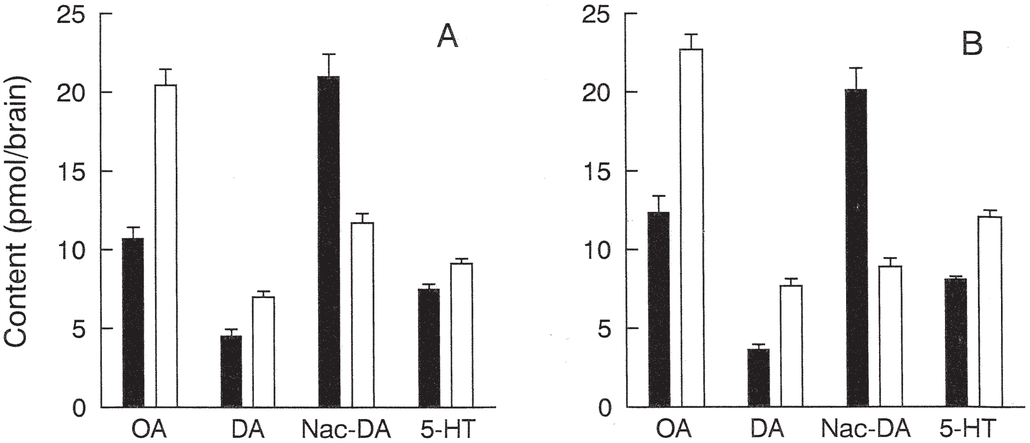

FIG. 6. The levels of biogenic amines in the brain. A, male crickets. B, female crickets. Solid bars, isolated crickets (n=38 in males and n=17 in females) ; open bars, crowded ones (n=25 in males and n=19 in females) ; mean±S. E. OA, octopamine; DA, dopamine; Nac-DA, N-acetyldopamine; 5-HT, 5-hydroxytryptamine.

ences between the isolated and the crowded were not observed both in males and females. The ratio of the level of biogenic amine to that of its metabolite indicates a magnitude of turnover rate, an index of neuronal activity in aminergic neurons. In this context, the ratio of the levels of dopamine to that of N-acetyldopamine showed noticeable differences. The ratios in the corpora allata and the corpora cardiaca were 3–7 despite the population density, whereas those in the brain of the crowded animal were markedly low compared to the isolated animal both in males and females (Fig. 6). Although not marked, a similar tendency was observed in the frontal ganglion, a part of the insect nervous system.

DISCUSSION

In the present study, we examined effects of population density by rearing crickets in two extreme conditions, completely isolated and exceedingly crowded. The crowded condition slowed the rate of body weight increase. In the adult stage, the average body weight of the crowded was 0.58 ±0.02 g and that of the isolated was 1.06±0.02 g. The crowded condition also caused the delay of imaginal moult. Although the number of ecdysis was not counted, the period between consecutive instars possibly became longer, or ecdysis were less frequent in the crowded. The crowded condition further suppressed black pigment synthesis, and decreased aggressiveness. Since *Gryllus bimaculatus* usually lives solitary in the field, we consider that the results yielded from the isolated animals reflect normal biological features in this species.

In addition to suppression of growth, black pigmentation and aggressiveness, the contents of biogenic amines were modulated by the crowded condition, that is, higher level of 5-HT and octopamine in the corpora allata and the corpora cardiaca, respectively, and suppression of the brain dopaminergic system. Since these results were coincidentally observed both in males and females, the changes in the aminergic system were probably induced by the crowded condition.

The corpora allata, which secretes juvenile hormone, are innervated by the nervi corporis allati I (NCA I). The NCA I is originated from the brain, and also includes a prominent tract composed of 5-HT-immunoreactive axons in the cricket, *Teleogryllus commodus* [16]. Meanwhile, juvenile hormone is known to retain larval forms and also reduce black pigmentation. These phenomena induced by juvenile hormone are comparable to the effects of crowded condition, the delay of imaginal moult and suppression of black pigment synthesis, suggesting that the crowded condition stimulates secretion of juvenile hormone. Secretion of juvenile hormone is usually suppressed in the final ecdysis, but in the crowded condition, secretion may not suppressed and juvenile hormone effected to the development and body color. If it is true, then, 5-HT should be involved in facilitatory control of juvenile hormone secretion. Although octopamine was reported to enhance release of juvenile hormone through cAMP in the corpora allata of *Locusta migratoria* [7], the present result, inconsistency of octopamine levels between males and females, seems to indicate that octopamine in the corpora allata has minor roles in the effects of population density.

Compared to the corpora allata, octopamine in the corpora cardiaca seems to have an important role in control of secretory activity in the crowded crickets. Since the release of adipokinetic hormone from the corpora cardiaca is considered to be under the synaptic control of axons in the nervi corporis cardiaci II [13], octopamine may control secretion of this hormone in the crowded. In the natural habitat, *G. bimaculatus* rarely showed mass migration by flight [17]. Despite a lack of experimental evidences, it is possible that, in the crowded condition, octopamine is stored in the nerve endings in the corpora cardiaca and is utilized if the crickets migrate in mass. Adipokinetic hormone is known to be released by a decrease in hemolymph trehalose that is induced by flight, and octopamine is attributable to lipid metabolism in insects [14].

The crowded condition apparently suppressed the activity of brain dopaminergic system, particularly its turnover rate, both in males and females. As are the isolated males, the isolated females are more aggressive than the crowded (unpublished observation, M. Iba and T. Nagao), so that the brain dopaminergic system may be related to control of aggressiveness in the crickets. The presence of such aminergic activating systems in the brain is well established in the vertebrate brains, however, in the higher invertebrates, clarification of the presence of activating systems requires further extensive investigation.

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