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Source: Zoological Science, 13(3) : 449-454

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.13.449

ZOOLOGICAL SCIENCE **13**: 449–454 (1996)

Species-Specificity in the Action of Big and Small Prothoracicotropic Hormones (PTTHs) of the Swallowtail Butterflies, *Papilio xuthus*, *P. machaon*, *P. bianor* and *P. helenus*

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ABSTRACT—To investigate whether four *Papilio* species, *Papilio xuthus*, *P. machaon*, *P. bianor* and *P. helenus*, have two molecular forms of the prothoracicotropic hormones (PTTHs), referred to as big- and small-PTTHs, the PTTHs were extracted and fractionated from their pupal brains. The activating ability of big- and small-PTTH fractions was examined by the *in vitro* assay using the prothoracic glands (PGs) of 2-day-old 5th-instar larvae of their own and several other papilionid species.

Big- and small-PTTH fractions activated the larval PGs of their own species to increase the ecdysteroid secretion *in vitro*. The doses of small-PTTH fractions for activating the larval PGs were 8- to 10-times larger than those of big-PTTH fractions.

The big- and small-PTTH fractions as well as those of *P. machaon* activated the PGs of 2-day-old 5th-instar larvae of several heterogeneous papilionids, but the activating ability did not always decrease with the distance of the genetic (or phylogenetic) relationships.

The results indicate that *P. machaon*, *P. bianor* and *P. helenus* may have two molecular forms of the PTTHs, both of which activate the larval PGs of the same species *in vitro* as in the case of *P. xuthus*. The big- and small- PTTHs of *P. xuthus* as well as those of several other *Papilio* species may retain an ability to activate the 5th-instar larval PGs of numbers of heterogeneous papilionids *in vitro*.

INTRODUCTION

Cerebral neuropeptides, prothoracicotropic hormones (PTTHs), activate the prothoracic glands (PGs) to secrete ecdysteroids which are essential for growth and metamorphosis in insects (Fukuda, 1944; Williams, 1946; Ishizaki and Ichikawa, 1967; Chino *et al.*, 1974; Warren *et al.*, 1988).

The silkworm, *Bombyx mori*, has two molecular forms of the PTTHs, referred to as *Bom*-PTTH and bombyxin (30 kD and 5 kD) (Yagi *et al.*, 1995), both of which activate the PGs from 2-day-old 4th-instar larvae of the same species *in vitro* (Fujimoto *et al.*, 1991; Kiriishi *et al.*, 1992). However, the former is thought to be the only PTTH of *B. mori*, since the latter failed to cause the PGs to secrete ecdysteroids *in vivo* (Kobayashi and Yamazaki, 1966; Ishizaki *et al.*, 1977; Ishizaki *et al.*, 1983; Nagasawa *et al.*, 1984; Kiriishi *et al.*, 1992).

Two molecular forms of the PTTHs, named the big- and small-PTTHs in *Manduca sexta* (Bollenbacher *et al.*, 1984), were demonstrated to exist in several other lepidopterans, such as *Polygonia c-aureum*, *Papilio xuthus* and *Mamestra brassicae*. The big- and small-PTTHs extracted from the pupal brains of those lepidopterans activated the PGs of 2-day-old last-instar larvae of their own species *in vitro* (Endo *et al.*, 1990; Fujimoto *et al.*, 1991).

The activating ability of the small PTTHs, quantified by the *in vitro* assay using the larval PGs of their own species, was far lower than that of the big-PTTHs in several lepidopterans. However, the small-PTTHs showed cross-reactivity to the PGs of last-instar larvae of several heterogeneous species whose range may be wider than the big-PTTHs (Bollenbacher *et al.*, 1984; Endo *et al.*, 1990; Fujimoto *et al.*, 1991).

In numbers of papilionids, the distance of the genetic (or phylogenetic) relationships was attempted to be evaluated on the basis of the egg hatchability, adult formation and F_1 fertility of interspecific hybrids (Ae, 1988).

Our study was intended to investigate whether three *Papilio* species, *P. machaon*, *P. bianor* and *P. helenus*, have two molecular forms of PTTHs activating the PGs of their own species *in vitro*. Then, the activating ability of the big- and small-PTTHs was examined by an *in vitro* assay using the

PGs of 5th-instar larvae of three or four heterogeneous papilionids.

MATERIALS AND METHODS

Animals

Five papilionid species, *P. xuthus*, *P. machaon*, *P. bianor*, *P. helenus* and *Byasa alcinous*, collected in the towns of Yamaguchi and Hofu were used.

Larvae of papilionids were reared in containers of transparent plastic and subjected to a long-day condition of alternating 16-hr light and 8-hr dark periods at 20°C or 25°C. In the photophase, light intensity was provided at about 400 lux on rearing containers with two 20-W white fluorescent tubes which were controlled by a 24-hr time-switch.

Larvae of *P. xuthus*, *P. bianor* and *P. helenus* were fed on fresh leaves of *Fagara ailanthoides* and those of *P. machaon* and *B. alcinous* were fed on leaves of *Glehria littleralis* and *Aristolochia debilis*, respectively.

Extraction of PTTHs

Brains were obtained from 0-day-old pupae of four *Papilio* species, *P. xuthus*, *P. machaon*, *P. bianor* and *P. helenus*, by dissection in 0.9% NaCl. A batch of 250 brains was homogenized with a glass homogenizer in 5 ml of total volume of acetone on ice, washed 3 times in 7.5 ml of total volume of 80% ethanol and extracted with 1.8 ml of Grace's medium for 4 min at 95°C. The extracts were cooled rapidly and added to 0.7 ml of fresh Grace's medium to provide crude PTTH extracts. At each step, insoluble materials were removed by a centrifugation at 12,000×*g* for 20-30 min.

Separation of big- and small-PTTHs

Each crude PTTH extract made from 0-day-old pupae of *Papilio* species was put in a tube with an ultrafilter passing smaller molecules than 10kD (Ultrafree C3LGC00, Millipore, Tokyo, Japan) and centrifuged at 3,000×*g* for 4-5 hr at 4°C. The filtrates were used as small-PTTH fractions, whereas the residues were washed twice with 1.0 ml of Grace's medium and served as big-PTTH fractions.

Quantification of the action of big- and small-PTTH fractions on the PGs

The activating ability of big- and small-PTTH fractions was quantified by the *in vitro* assay. The pairs of the PGs obtained from 2-day-old 5th-instar larvae of papilionids by dissection in saline (Wyatt, 1961) were washed in Grace's medium for 30-60 min. One of the pair was incubated in Grace's medium alone (50 μl) and the contralateral was incubated in Grace's medium (50 μl) with one of the assaying samples at 25°C. After a 2-hr incubation, the PGs were removed and the amount of ecdysteroids secreted into the incubation medium was quantified by the radioimmunoassay (RIA). The activation ratio (Ar) showing the amount of ecdysteroids secreted by the experimental PG divided by that secreted by the control PG was obtained by the incubations of 5-6 PG pairs (Bollenbacher *et al.*, 1984). Fractions recording Ar-values larger than 2.0 (or 3.0 in some cases, *t*-test: $P>0.01$) were judged to show PTTH-activity.

Assay of ecdysteroids

The titer of ecdysteroids in each sample (5 μl or 20 μl) was measured as a corresponding response to ecdysone (Sigma Chemical Co., St. Louis, USA), using the RIA method (Borst and O'Conner, 1972). Antiserum raised against 20-hydroxyecdysone (Rhoto Pharmaceutical, Osaka, Japan) was obtained from the Meguro Institute, Osaka, Japan. This antiserum exhibits reactivity against 20-hydroxyecdysone approximately 5-times stronger than that against ecdysone and 3-dehydroecdysone. Radioactive ecdysone (23, 24-^{3}H(N))-ecdysone (2,960 GBq/mmol) was obtained from New England Nuclear, Boston, USA. A lower limit of detection of ecdysone by this RIA method was 2.5 pg/20 μl.

RESULTS

The activating ability of big- and small-PTTHs to the PGs of 5th-instar larvae of four Papilio *species*

To examine whether four *Papilio* species have two molecular forms of the PTTHs activating the 5th-instar larval PGs of their own species *in vitro*, brains were obtained from 0-day-old non-diapause pupae of four *Papilio* species, *P. xuthus*, *P. machaon*, *P. bianor* and *P. helenus*, by dissection in 0.9% NaCl and stored at -85°C. Batches of 250 brains were extracted with Grace's medium and extracts were filtrated with an ultrafilter to obtain big- and small-PTTH fractions. The activating ability of each fraction was quantified by the *in vitro* assay using the PGs of 2-day-old 5th-instar larvae of their own species at 25°C. The doses of big- and small-PTTH fractions added in the incubation medium (50 μl) were changed from 1/8 to 4 and 1/4 to 8 brain-equivalents, respectively.

All PGs of 5th-instar larvae of four *Papilio* species secreted ecdysteroids at the rate of 7-15 pg/gland/hr by a 2-hr incubation in Grace's medium alone. The rate of ecdysteroid secretion was increased by adding big- or small-PTTH fractions of their own species into incubation medium. The rate of secretion reached 250-750 pg/gland/hr at its maximum.

Ar-value of each fraction showed the activating ability to the PGs examined *in vitro*. The Ar-values reached maximum (Ar 4-8) when the PGs of 2-day-old 5th-instar larvae of four *Papilio* species were incubated in Grace's medium (50 μl) with big-PTTH fractions of 1-2 brain-equivalent. Half maximum activation was obtained by the fraction doses of 1/4 to 1/2 brain-equivalent, respectively (Fig. 1 and Table 1).

Small-PTTH fractions also activated the 5th-instar larval PGs of their own species (Ar 4-8). The maximum Ar-value obtained by the small-PTTH fraction of each species was almost the same value as that obtained by the big-PTTH fraction. However, for inducing a detectable response, each small-PTTH fraction required an 8- to 10-times larger dose (4-8 brain-equivalents) than that for the big-PTTH fractions (1/2-1 brain-equivalent), respectively (Fig. 1 and Table 2).

The results indicated that four *Papilio* species, *P. xuthus*, *P. bianor*, *P. helenus* and *P. machaon*, have big- and small-PTTHs, both of which activate the 5th-instar larval PGs of their own species *in vitro*. However, the doses of small-PTTH fractions which are required for activating the PGs *in vitro* seem to be far larger than those required for the big-PTTH fractions.

The activating ability of big- and small-PTTHs of P. xuthus *to the PGs of 5th-instar larvae of heterogeneous* Papilio *species*

To know whether big- and small-PTTHs of *P. xuthus* activate the PGs of heterogeneous papilionids, pairs of the PGs were isolated from 2-day-old 5th-instar larvae of *P. bianor*, *P. helenus*, *P. protenor* and *B. alcinous* and were incubated for 2 hr in Grace's medium with or without big- and small-PTTH fractions of *P. xuthus* at 25°C. The doses of big- and small-PTTH fractions were changed from 1/8 to 4 and from 1/

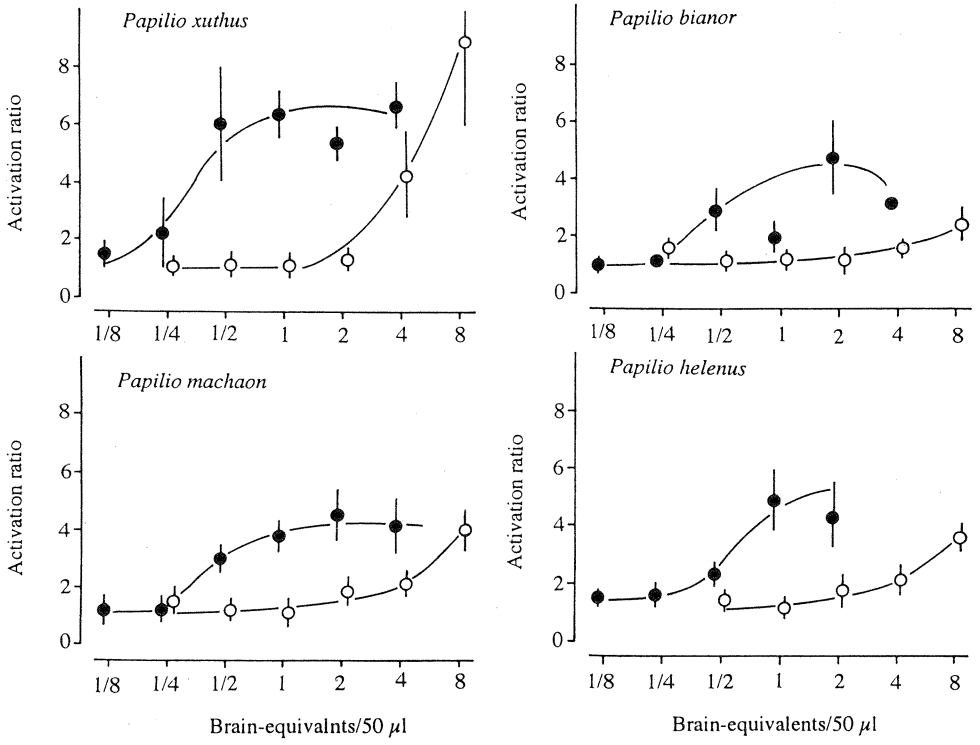

Fig. 1. The dose-dependent response of the 5th-instar larval prothoracic glands (PGs) of *P. xuthus*, *P. machaon*, *P. bianor* and *P. helenus* to big- and small-PTTH fractions of their own species. Solid and open circles show activation ratios of the big- and small-PTTH fractions, respectively. Each datum point is the mean with obtained by the incubations of 5-6 PG pairs.

Table 1. The activating ability of big-PTTH fractions to the 5th-instar larval PGs of their own and heterogeneous *Papilio* species

Insect species obtained big-PTTH fractions	Doses of big-PTTH fractions required for inducing a half maximum response in the PGs of the species indicated (brain-equivalent/50 μl)			
	P. xuthus	*P. bianor*	*P. helenus*	*P. protenor*
P. xuthus	1/4 - 1/2	1/4 - 1/2	1/4 - 1/2	1/4 - 1/2
P. machaon	1/2 - 1	1/4 - 1/2	1/4 - 1/2	not activated
P. bianor	1/2 - 1	1/2 - 1	1/4 - 1/2	not examined
P. helenus	1 - 2	1 - 2	1/4	not examined

Larvae and pupae were reared under 16L-8D at 25°C and the PGs were obtained from 2-day-old 5th-instar larvae at 12:00-16:00 (AZT).

Table 2. The activating ability of small-PTTH fractions to the 5th-instar larval PGs of their own and heterogeneous *Papilio* species

Insect species obtained small-PTTH fractions	Doses of small-PTTH fractions required for inducing a detectable or minimum response (Ar>2) in the PGs of the species indicated (brain-equivalent/50 μl)			
	P. xuthus	*P. bianor*	*P. helenus*	*P. protenor*
P. xuthus	4	4	not activated	8
P. machaon	4	1/4	1	1
P. bianor	8	1	1	not examined
P. helenus	not activated	1/2	2	not examined

Larvae and pupae were reared under 16L-8D at 25°C and the PGs were obtained from 2-day-old 5th-instar larvae at 12:00-16:00 (AZT).

4 to 8 brain-equivalents, respectively.

The 5th-instar larval PGs of *P. bianor*, *P. helenus*, *P. protenor* and *B. alicinous* secreted ecdysteroids at the rate of 10-16 pg/gland/hr in Grace's medium alone. The rate of ecdysteroid secretion was increased by adding *P. xuthus* big-PTTH fraction into the incubation medium. The maximum Ar-values recorded by the PGs of *P. bianor*, *P. helenus*, *P. protenor* and *B. alcinous* were 3.4, 4.0, 3.0 and 10.4 when the fraction doses were 1, 4, 2 and 1 brain-equivalents, respectively (Fig. 2 and Table 1).

A small-PTTH fraction of *P. xuthus* also activated the PGs of 3 (out of 4) heterogeneous papilionids *in vitro*. The doses of small-PTTH fraction required for inducing a detectable response were 2-8 brain-equivalents, which were 4- to 10-times larger than those for the big-PTTH fractions (Fig. 2) as was shown in the 5th-instar larval PGs of their own species (Fig. 1). By contrast, the PGs of *P. helenus* showed no response to the *P. xuthus* small-PTTH fraction (Fig. 2 and Table 2).

The results indicated that the big- and small-PTTHs of *P. xuthus* seem to have activating ability to the PGs of numbers of papilionids *in vitro*. But, the activating ability of the big- and small-PTTHs of *P. xuthus in vitro* seems to vary depending on the species of the PG donors.

The activating ability of big- and small-PTTHs of P. machaon *to the PGs of 5th-instar larvae of three heterogeneous* Papilio *species*

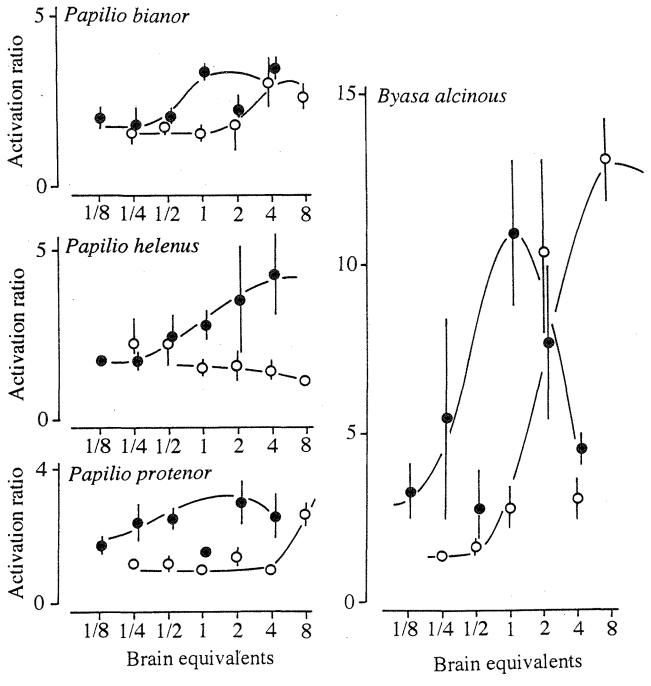

Fig. 2. Dose-dependent response of the 5th-instar larval prothoracic glands (PGs) of heterogeneous papilionids, *P. bianor*, *P. helenus*, *P. protenor* and *Byasa alcinous*, to big- and small-PTTH fractions of *P. xuthus*. Solid and open circles show activation ratios of the big- and small-PTTH fractions, respectively. Each datum point is the mean with standard error of the incubations of 5-6 PG pairs.

To examine whether big- and small-PTTHs of *P. machaon* activate the PGs of heterogeneous *Papilio* species, big- and small-PTTH fractions were made from a batch of 250 brains of 0-day-old non-diapause *P. machaon* pupae in the same manner as above, and the PTTH-activity (Ar-values) of the fractions was quantified by the *in vitro* assay using the PGs of 2-day-old 5th-instar larvae of *P. xuthus*, *P. bianor* and *P. helenus*.

All PGs secreted ecdysteroids at the rate of 8-14 pg/gland/hr in Grace's medium alone. The secretion rate was increased by adding one of the big- and small- PTTH fractions of *P. machaon* into the incubation medium (Fig. 2 and Tables 1 and 2). Ar-values of the big-PTTH fractions were 10.1, 2.4 and 2.8 at their maximum in the PGs of *P. xuthus*, *P. bianor* and *P. helenus*, respectively (Fig. 2). Half maximum Ar-values were obtained at the fraction doses of 1/2 to 1 brain-equivalent (50 μl) in the PGs of two (out of three) *Papilio* species (Table 1).

The *P. machaon* small-PTTH fraction also activated the PGs of three *Papilio* species *in vitro*. But, the activating ability (Ar-values) of the small-PTTH fraction in relation to the PGs of *P. bianor* and *P. helenus* was as low as that obtained by the big-PTTH fraction (Fig. 2 and Table 2).

The results indicated that the small-PTTH of *P. machaon* activates equally the 5th-instar larval PGs of three heterogeneous *Papilio* species *in vitro* but that the big-PTTH does not do so.

The activating ability of the big- and small-PTTHs of two Papilio *species,* P. bianor *and* P. helenus*, to the PGs of 5th-instar larvae of heterogeneous* Papilio *species*

Big- and small-PTTH fractions were prepared from a batch of 250 brains of 0-day-old *P. bianor* (or *P. helenus*) pupae and the PTTH-activity of the fractions was quantified by the *in vitro* assay using the PGs of 2-day-old 5th-instar larvae of *P. xuthus* and *P. helenus* (or *P. xuthus* and *P. bianor*). The big- and small-PTTH fractions of *P. bianor* activated the PGs of *P. xuthus* (Ar 4.5 and 2.8 at its maximum) and those of *P. helenus* (Ar 3.9 and 3.2 at its maximum) *in vitro*, respectively. A half maximum response of *P. bianor* big-PTTH fraction was obtained with a dose of 1/2-1 and 1/4-1-1/2 brain-equivalent in the PGs of *P. xuthus* and *P. helenus*, respectively (Table 1). While, the doses of *P. bianor* small-PTTH fraction which gave the PGs of *P. xuthus* and *P. helenus* a detectable response were 8 and 1 brain-equivalents, respectively (Table 2).

The *P. helenus* big-PTTH fraction activated the PGs of two heterogeneous *Papilio* species (Ar 3.4 and 4.9 at their maximum in the PGs of *P. xuthus* and *P. bianor*, respectively), whereas the *P. helenus* small-PTTH fraction activated the PGs of *P. bianor* (maximum Ar 4.1), but failed to activate the PGs of *P. xuthus in vitro*.

DISCUSSION

Three *Papilio* species, *P. machaon*, *P. bianor* and *P.*

helenus have two molecular forms of the PTTHs, both of which activate the PGs of 2-day-old 5th-instar larvae of the same species *in vitro*. Two molecular forms of the PTTHs found in these three *Papilio* species are thought to correspond to the big- and small-PTTHs demonstrated in *Manduca sexta* (Bollenbacher *et al.*, 1984) as was demonstrated in the case of the big- and small-PTTHs of *P. xuthus* (>15 kD and 4-5 kD) (Fujimoto *et al.*, 1991). The big-PTTH of *P. xuthus* activated the larval PGs of four heterogeneous papilionid species, *P. bianor*, *P. helenus*, *P. protenor* and *B. alcinous*, whereas the small-PTTH of *P. xuthus* activated the 5th-instar larval PGs of three (out of four) heterogeneous papilionid species. The activating ability of the PTTH to the PGs is thought to vary depending on the species of the PG donors examined. It was based on the doses of extracts required for inducing a half maximum response of the PGs (Tables 1 and 2). However, the Ar-values of the fractions as well as their dose-dependent response curves often varied when the PG donors were selected from several larval lots. The effects of PG donors on Ar-values and dose-dependent response curves could be excluded by carefully selecting the PG donors (4th-instar larvae) of the same size from the same larval lots. But, decrement of Ar-values occurred abruptly as observed in Fig. 2 (right) and Fig. 3 (top). The decrement is thought to be accidental, due, for example, to contamination with a poison(s) of the cervical gland by the dissection of the PGs, because it occurred occasionally even in the PGs of the same experimental group.

During the speciation, molecules of the PTTHs as well as the PTTH-receptors on the PGs may have changed to keep reactivity to each other, but the changes may occur with no relation to other species. In numbers of *Papilio* species, the big- and small-PTTHs were shown to retain the reactivity to the PGs of heterogeneous papilionids (Tables 1 and 2).

An examination of the distance of the genetic (or phylogenetic) relationships and grouping of species was attempted on the basis of the egg hatchability, adult formation and F_1 fertility of interspecific hybrids in numbers of *Papilio* species (Ae, 1988). A differentiation index (D-index) was set up to show the distance of genetic relationships. The D-indexes towards *P. machaon* were estimated to be 55, 59, 66 and 72 in *P. helenus*, *P. protenor*, *P. bianor* and *P. xuthus*, respectively.

Differences in the molecules of the PTTHs and their receptors may become larger, but the reactivity of the PTTHs to the PGs of heterogeneous species may not always decrease with the distance of the genetic relationships (or with the values of D-indexes) in *Papilio* species inhabiting Japan. That is, the big-PTTH of *P. machaon* exhibits far stronger activating ability against the PGs of *P. xuthus* (D-index 72) than that against the PGs of *P. bianor* (D-index 66) and *P. helenus* (D-index 55) (Fig. 3). While, the big-PTTH of *P. xuthus* showed a stronger activating ability to the PGs of *B. alcinous* than that to the PGs of their own and other *Papilio* species (D-index 0 and 100) (Fig. 2). However, two *Papilio* species whose big PTTHs failed to activate the PGs of the others, *P. xuthus* and *P. helenus*, may not produce their interspecific hybrid undergoing the post embryonic development.

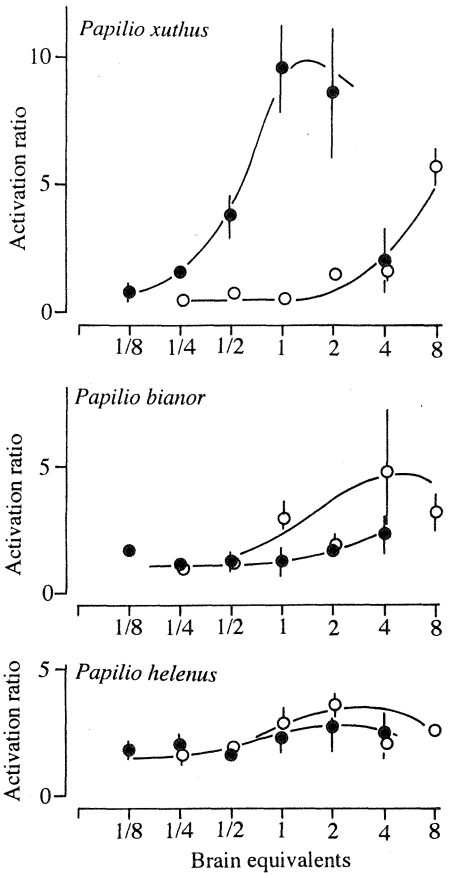

Fig. 3. The dose-dependent response of the 5th-instar larval prothoracic glands (PGs) of heterogeneous *Papilio* species, *P. xuthus*, *P. bianor* and *P. helenus*, to big- and small-PTTH fractions of *P. machaon*. Solid and open circles show activation ratios of the big- and small-PTTH fractions, respectively. Each datum point is the mean with standard error of the incubations of 5-6 PG pairs.

Here, we were not able to provide any evidence about molecules of the PTTHs and their receptors in *Papilio* species. But, that may emerge following a course of molecular biological study about the PTTHs and PTTH-receptors of *Papilio* species.

ACKNOWLEDGMENT

We thank Dr. D. Le Sage for his critical reading of this manuscript. This work was supported in part by the Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (No. 06640881).

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(Received November 24, 1995 / Accepted March 29, 1996)