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A novel low molecular weight ecdysiotropin in post-diapause, pre-hatch eggs of the gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae)

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Abstract

Extracts of post-diapause, pre-hatch eggs of the gypsy moth, *Lymantria dispar* L. were examined for prothoracicotropic hormone (PTTH)-like activity using an in vitro assay involving last-instar prothoracic glands (PGs). The eggs were extracted in water, eluted from a low-pressure C_{18}-silica cartridge in 60% acetonitrile, and fractionated on a high-performance, size-exclusion column. The primary ecdysiotropic activity eluted with an estimated molecular weight of 2.1 kDa far below the 4–7 kDa size determined for the low molecular weight PTTHs (bombyxins). Dose-response analysis revealed that the maximum activation was reduced by 75% by organic solvent extraction, but the remaining activity retained the ability to maximally activate the PGs 10-fold in vitro. At least some of the ecdysiotropic activity in the post-diapause, pre-hatch egg is localized in the brain of the pharate larva, and this activity increases dramatically prior to hatch when eggs are incubated at 25°C.

Keywords: Embryo; Prothoracicotropic hormone; Diapause; Univoltine; Ecdysteroid

1. Introduction

The function of the brain neuropeptide, prothoracicotropic hormone (PTTH) in the insect larva and pupa is to stimulate ecdysteroid production by the prothoracic glands (PGs), which in turn is converted to 20-hydroxyecdysone by peripheral tissues, resulting in the onset of molting and metamorphosis [1–4]. In the three most thoroughly studied insect species with regard to PTTH, the commercial silkworm, *Bombyx mori*, the tobacco hornworm, *Manduca sexta* and the gypsy moth, *Lymantria dispar*, PTTH exists as part of a family of ecdysiotropic neuropeptides having two molecular weight ranges:

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4–7 kDa for small PTTH (bombyxin) and 11–27 kDa for large PTTH [5]. In many insect species, the lack of release of brain ecdysiotropin has been associated with the induction of diapause in the larva or pupa [6–10]. The study of Bell et al. [11] on *L. dispar* egg diapause suggests that ecdysiotropin titers rise as the embryo matures around day 15 and fall prior to the onset of diapause. Following differentiation to the pharate larval stage, the larva enters an obligatory diapause [12] on day 20, which is marked by a 10-fold decrease in oxygen consumption [13]. Recent evidence in a number of insect species indicates that new synthesis of ecdysteroids may occur during embryogenesis (see review [14]), but their role in embryonic diapause termination remains unknown. Hence, knowledge of the titer, localization, and forms of ecdysiotropins present during embryogenesis and pharate larval development is important in elucidating the role of the ecdysiotropins in embryonic development, diapause, and diapause termination.

Studies with *B. mori* [15–17] and *M. sexta* [18] have demonstrated ecdysiotropic activity in embryonated eggs and embryos, respectively. More recent studies with *L. dispar* ([5,11,19–21] and other references cited therein) have shown similar activity, both in vitro and in vivo, in extracts of whole eggs containing developing embryos and pharate larvae. Ecdysiotropic activity was most clearly demonstrated in *L. dispar* eggs about to hatch [20,21]. In this case, the majority of the activity was sensitive to denaturation by heat or organic solvent extraction [20], but this denaturable activity appeared due to 3-oxoecdysteroid reductase activity present in the crude egg extracts which enhanced the quantity of immunoreactive ecdysteroids detected by the radioimmunoassay (RIA) [22]. The remaining stable, ecdysiotropic activity was protease sensitive [20]. We have investigated further this activity in post-diapause, pre-hatch eggs of *L. dispar* and show in the present paper a novel low molecular weight form of ecdysiotropin. At least some of this activity is associated with the head region of the embryo where brain activity shows a previously undemonstrated increase prior to hatch.

2. Materials and methods

2.1. Experimental animals

The colony was derived from a New Jersey population in 1967, designated 'NJ', and reared on a high wheat germ diet according to Bell et al. [13]. Eggs were maintained at 5–7°C for 5–6 months to break diapause, and newly hatched larvae were maintained (10 larvae/180 ml cup) at 25°C, 50–60% relative humidity, and a photoperiod of 16:8 (L/D) h. Late fourth instars were staged according to Thyagaraja et al. [21], and newly ecdysed females designated Day 1 (i.e., L5d1). Under these rearing conditions the 5th (final) instar lasts 11–12 days. The few larvae destined to become 6th instars were not used. Wild eggs were obtained in March and April, 1988 at Harpers Ferry, MD, designated 'HF', and maintained at 7°C until a few days prior to hatch when they were frozen at −20°C.

2.2. Egg extraction

De-hairing of egg masses and extraction of eggs with a Polytron homogenizer (Brinkman Instruments, NY) in HPLC-grade water or organic solvent (90% methanol (CH_3OH), 0.1% trifluoroacetic acid (TFA), Aldrich Chemical Co., Milwaukee, WI) were done, in general, by the methods of Masler et al. [20].

Exceptions were that eggs were extracted in larger volumes of HPLC-grade water with a larger generator (i.e., PTA-20 or PTA-36/2M) while maintaining the original ratio of the weight of the eggs to the volume of HPLC-grade water (100 mg/ml). This had no effect on the final activity. Extracted material was vacuum dried, stored at −20°C and subsequently resolubilized in Grace's medium (Gibco, Grand Island, NY) at appropriate dilutions for assay. The

data are recorded as mg egg equivalents/μl since large numbers of eggs were weighed at one time. The number of individual eggs was not determined. No loss of the active material was assumed during purification. Equivalent weights are based on the weight prior to extraction. One post-diapause, pre-hatch egg of *L. dispar* weighs ca. 700 μg.

2.3. In vitro assay

PG assays were performed according to the methods of Kelly et al. [23]. Essentially, individual PGs were incubated for 2 h in 25 μl drops of Grace's medium to allow ecdysteroid secretion to drop to basal levels, then the PGs were further incubated for 2 h with egg extracts in Grace's medium (left PG) or fresh Grace's medium (right PG). An activation ratio (A_r) was determined by dividing the amount of ecdysteroid secreted by the extract-activated PG (i.e., experimental) by that of the contralateral gland in Grace's medium (i.e., control). For some experiments, a net synthesis assay was employed (requiring fewer PGs) by subtracting the amount of ecdysteroid secreted during the 3rd h of incubation in Grace's medium from the amount secreted during the 4th h in the presence of egg extracts [23].

2.4. Chromatography

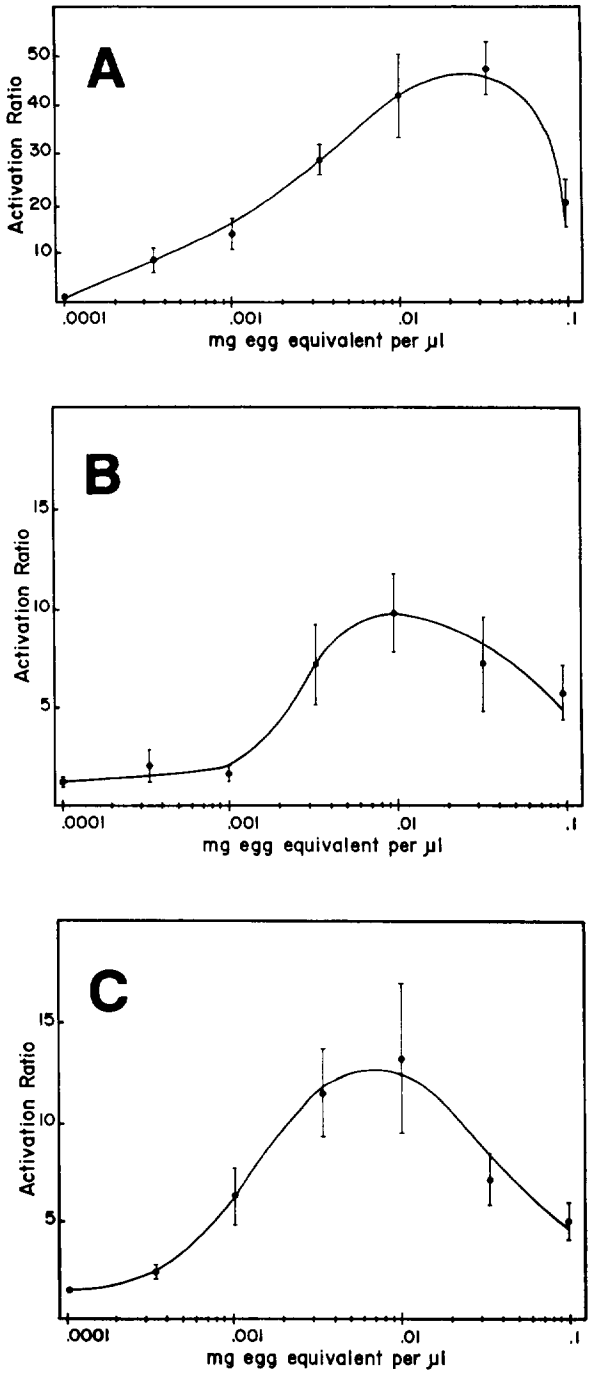
Egg extracts, previously extracted in HPLC-grade water, vacuum-dried and stored at −20°C, were resolubilized and fractionated by methods [19] modified from those developed for postembryonic PTTH fractionation [24]. Basically, 1–2 g egg equivalents of H_2O-extracted material were resolubilized in 3.0 ml of HPLC-grade water and passed through a C_{18} SEP-PAK cartridge (Waters Assoc., Milford, MA) and the 60% acetonitrile (CH_3CN) eluate collected according to Masler et al. [25] and vacuum dried. For high-performance, size-exclusion chromatography (HP-SEC), SEP-PAK fractions were resolubilized in 100 μl 40% CH_3CN, 0.1% TFA, spun at 12,000 *g* for 1 min, fractionated in the same solvent on a tandem Waters I-125 HP-SEC column system at 1 ml/min (room temperature), 0.5 ml fractions collected, vacuum dried and stored at −20°C until assay following resolubilization in Grace's medium. Molecular weight markers were purchased from Sigma Chemical Co., St. Louis, MO except for little gastrin which was purchased from Peninsula Laboratories, Belmont, CA.

2.5. Ecdysteroid analysis

Media from PG incubations were either assayed immediately for ecdysteroid content, or stored at −20°C until needed. Ecdysteroid activity present in tissue extracts or column fractions was subtracted from ecdysteroid activity present in the medium of experimental glands before the calculation of A_r values or the determination of net synthesis. Samples were assayed for ecdysteroid content by RIA according to Kelly et al. [26]. The antibody was a gift from W.E. Bollenbacher, University of North Carolina, Chapel Hill, and its affinity for various ecdysteroids has been characterized [27]. [23,24-^{3}H(N)]Ecdysone was obtained from NEN Research Products (Boston, MA, 50–80 Ci/mmol) and ecdysone from Sigma Chemical Co. (St. Louis, MO).

3. Results

Since our current studies were performed primarily with egg masses collected in the wild (designated HF strain), and Masler et al. [20] had used a laboratory strain (designated NJ strain), we initially repeated their analysis to verify the extraction procedure and the presence of ecdysiotropic activity in the HF eggs. Extraction of these pre-hatch eggs in HPLC-grade water following the procedure of Masler et al. [20] gave similar dose-response results when analyzed in vitro on PGs from L5d5 females (Fig. 1A). The dose-response kinetics for HF eggs revealed a maximum activation ($A_{r,max} \approx 40$) at 0.010 mg egg eq./μl. However, the activation range



was broader with a smaller ED_{50} at a concentration of 0.002 mg egg eq./µl versus 0.004 mg egg eq./µl for the NJ eggs used by Masler et al. [20].

Further comparison of the extraction methods using acid-methanol (90% CH_3OH, 0.1% TFA) showed that the activity was retained (Fig. 1B), when compared at the ED_{50} dose of 0.002 mg egg eq./µl, but the $A_{r,max}$ was reduced to 25% comparable to the 35% reported by Masler et al. [20]. Since this acid-methanol-extractable activity was compatible with the solvent systems used for SEP-PAK extraction, we examined the recovery following elution from a C_{18} SEP-PAK cartridge (Fig. 1C). The dose-response curve for this SEP-PAK-extracted material was comparable to that of the acid-methanol extracted material (Fig. 1B), suggesting that the activity soluble in acid-methanol could be fractionated by SEP-PAK procedures [25].

Using material that had been partially purified by SEP-PAK extraction (resulting in 99.4% total purification by weight), egg samples were analyzed by HP-SEC fractionation followed by in vitro PG assay of the fractions at 100 mg egg eq. per fraction. Activity was revealed primarily in a low molecular weight region much less than the 6.5 kDa standard (Fig. 2). Fraction 12, the most active fraction, was further analyzed by dose-response (Fig. 3), revealing an $A_{r,max} \approx 10$, comparable to the SEP-PAK-extracted starting material, but a greatly reduced $ED_{50} \approx 0.02$ mg egg eq./µl, suggesting considerable

Fig. 1. In vitro response of day 5, 5th-instar *L. dispar* female prothoracic glands to post-diapause, pre-hatch egg extracts. Egg extracts were from wild 'HF' eggs. (A) H_2O-extract, vacuum dried. (B) Further extracted in acid-methanol (90% CH_3OH, 0.1% trifluoroacetic acid (TFA)). (C) Extract from (A) following elution from a C_{18}-silica cartridge with 60% acetonitrile (CH_3CN). Doses are expressed as milligram egg equivalent per microliter of incubation medium. The ratio of ecdysteroid in post-incubation experimental gland medium to that in post-incubation control gland medium (activation ratio, A_r) is used as a measure of ecdysiotropic activity (see Materials and methods). Each datum point represents the average A_r of 6–12 replicates ± S.E. Points without standard error bars had errors smaller than the point.

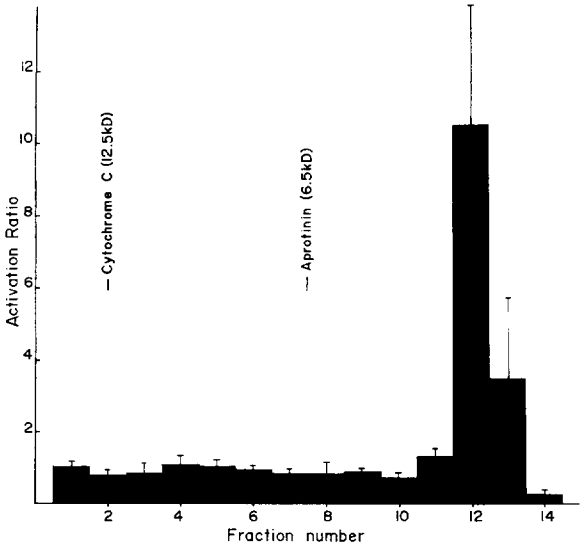


Fig. 2. Size-exclusion fractionation of *L. dispar* egg extract from HF eggs. 200 mg egg eq. were loaded per column and half of each fraction used for assay. Following resolubilization in Grace's medium, fractions were analyzed by in vitro incubation with prothoracic glands of day 5, 5th-instar females. Activation ratios were determined as described in Fig. 1. Molecular weight markers were carbonic anhydrase (29 kDa) which eluted before the first fraction was collected, cytochrome *c* (12.5 kDa), and aprotinin (6.5 kDa). Each data point represents the means ± S.E. of 3 replicates.

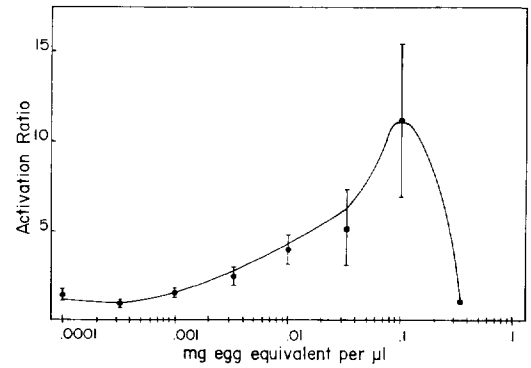


Fig. 3. In vitro response of day 5, 5th-instar female prothoracic glands to dilutions of fraction # 12 from Fig. 2. Half of fraction # 12 (100 mg egg equivalents) was redissolved in 300 µl of Grace's medium and dilutions prepared as indicated. Activation ratios were determined as described in Fig. 1. Each data point represents the means ± S.E. of 3 replicates.

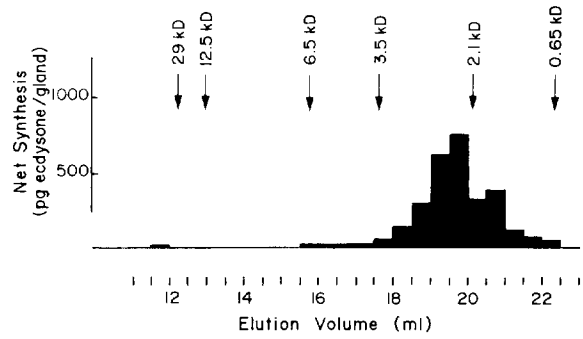


Fig. 4. Size exclusion fractionation of *L. dispar* egg extract from HF eggs. 100 mg egg eq. were loaded per column. Following resolubilization in Grace's medium, fractions were analyzed by in vitro incubation with prothoracic glands of day 5, 5th-instar females. Net synthesis was determined by subtracting 3rd-h synthesis in Grace's medium from 4th-h synthesis in Grace's medium plus fractionated egg extract. Molecular weight markers were carbonic anhydrase (29 kDa), cytochrome *c* (12.5 kDa), aprotinin (6.5 kDa), insulin chain B (3.5 kDa), little gastrin (2.1 kDa), proctolin (0.65 kDa).

loss of activity. Reanalysis by HP-SEC, incorporating lower molecular weight standards to more accurately assess the molecular weight and using the net synthesis assay (see Materials and methods) for comparison to previous results with L5d5 *L. dispar* brains [23], resulted in an estimated molecular weight of 2.1 kDa (Fig. 4).

For studies to localize the activity in whole eggs containing mature larvae (Fig. 5A,B), NJ strain embryos were dissected in *Bombyx* saline [28] and divided into head and thorax/abdomen regions. Dose-response analyses revealed that the head region contained greater activity than the thorax/abdomen region (4-fold greater at 4 tissue eq./25 µl), although maximum activation was not achieved so that ED_{50} values could not be obtained for accurate quantitation of the ecdysiotropic activity. Further analysis of the ecdysiotropic activity in brains from pharate larvae maintained in diapause conditions

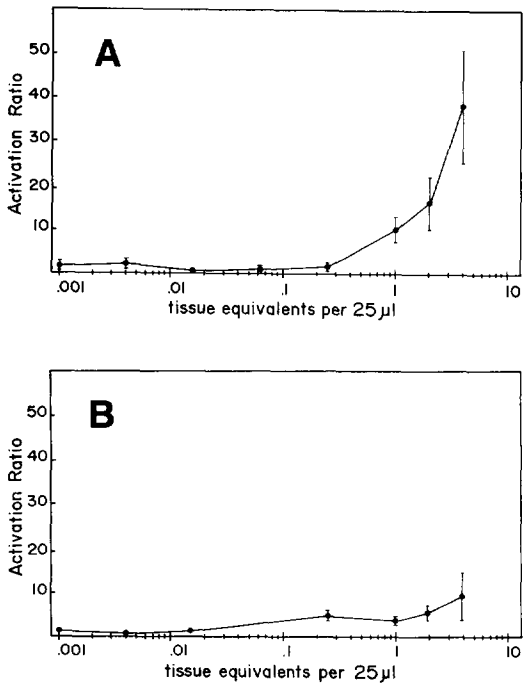


Fig. 5. In vitro response of day 5, 5th-instar female prothoracic glands to *L. dispar* pharate larval extracts. Mature (pre-hatch) NJ larvae were dissected in *Bombyx* saline [28] and heads (A) and thorax/abdomens (B) were pooled from 50 animals, homogenized in Grace's medium, and supernatants from a 1 min 12,000 *g* spin were removed and diluted as indicated. Assay conditions were as described in Fig. 1 legend. Each data point represents the means ± S.E. of the A_r of 3 replicates. Points without standard error bars had errors smaller than the point.

(5–7°C) for ca. 150 days showed activity in vitro at 2.0 brain equivalents/25 µl, but this activity increased considerably following development at 25°C for 4 days (Table 1), being evident at 0.25 brain equivalents/25 µl.

4. Discussion

The data indicate that mature, post-diapause *L. dispar* eggs, collected in the wild at Harpers Ferry, MD (HF strain), contain ecdysiotropic activity. Furthermore, the data show similarities to data obtained from a laboratory-reared strain (NJ strain; [20]) originally derived from a New Jersey population in 1967 [13]. When examined by dose-response analysis (Fig. 1A), a crude H_2O-extract of HF eggs gave maximum activation at 0.010 mg egg eq./µl, identical to the crude H_2O-extract of mature, post-diapause NJ eggs [20]. The HF eggs, however, showed an extended activation range (100-fold) with an ED_{50} at a concentration of 0.002 mg egg eq./µl (2-times lower than with NJ eggs). Organic solvent extraction of HF eggs reduced the $A_{r,max}$ by ≈75% (Fig. 1B), again comparable to the data of Masler et al. [20] for NJ eggs. The reduction in $A_{r,max}$ is apparently due to the presence of a 3-oxoecdysteroid reductase in crude egg extracts that is unstable to heat and organic solvent extraction [19,20,22]. This

Table 1

Activation of brain ecdysiotropic activity in brains of pre-hatch *L. dispar* larvae

Brains per 25 µl	Treatment at 25°C	
	–	+
0.125	0	0
0.25	0	53 ± 51
0.5	0	309 ± 286
1.0	0	566 ± 189
2.0	21 ± 12	398 ± 224
4.0	115 ± 54	470 ± 263

Newly laid eggs from mated females were maintained at 25°C, 16:8 light/dark cycle for 30–35 days and then at 5–7°C for ca. 150 days to allow diapause development as described [13]. Following this treatment, brains were either dissected immediately in *Bombyx* saline and frozen at −80°C until use (−) or maintained at 25°C for 4 days prior to dissection when the eggs in each egg mass begin to hatch (+). Brain extracts were prepared in Grace's medium and tested in the net synthesis assay as described in Fig. 4 following placement in boiling water for 2 min and centrifugation at 12,000 *g* for 1 min. The data are presented as ecdysone equivalents per gland and represent the means ± S.E. for 3–6 replicates.

enzyme functions to convert PG-produced 3-dehydroecdysone, barely detectable by our RIA, into RIA-detectable ecdysone [22]. Each sample containing non-denatured egg extract would thus give enhanced RIA results.

To remove the reductase activity and extract the ecdysiotropic activity, HF egg extract was further analyzed by fractionation with organic solvents on a low pressure C_{18}-silica cartridge (Fig. 1C) followed by HP-SEC (Fig. 2). When analyzed in this manner, substantial activity was obtained in fractions eluting after the lowest molecular weight standard (aprotinin, 6.5 kDa), thus indicating ecdysiotropic activity in material of apparent molecular weight lower than that generally observed for brain extracts from larvae and pupae of *L. dispar* and other lepidopterans. The discovery of such low molecular weight ecdysiotropic activity is not without precedent since low molecular weight ecdysiotropic activity was previously demonstrated in last-instar larval *L. dispar* [23] and European corn borer, *Ostrinia nubilalis* brains [10]. The partially purified ecdysiotropic activity from C_{18} reverse phase stimulates the prothoracic glands in a dose-dependent manner (Fig. 3) and has an estimated molecular mass of 2.1 kDa (Fig. 4). These findings are in contrast to the studies of Chen et al. [15,16] and Fugo et al. [17] with *B. mori* eggs in which they demonstrated large (20–30 kDa) and small (4–5 kDa) forms of ecdysiotropin similar to that found in *B. mori* larvae and pupae. However, the methods employed by these investigators were considerably different from ours, and they began with considerably more starting material (50 g versus 100 mg). Further analyses will be necessary to define the reasons for these differences.

The exact location of the ecdysiotropic activity in the post-diapause, pre-hatch egg of *L. dispar* has not been determined. However, our initial experiments indicate that at least some of the activity in the mature embryo is localized in the head region (Fig. 5), although the possibility that the head region may contain considerably more reductase activity than the thorax/abdomen has not been ruled out. Dorn et al. [18] also found considerable ecdysiotropic activity in the embryonic head of maturing *M. sexta* embryos and further localized it to the brain. Comparison of the dose-response curves of isolated *L. dispar* heads versus whole eggs containing mature embryos suggests that considerable activity resides outside the embryo, although, again, this could be due to reductase activity. Assuming that the ED_{50} for isolated NJ heads is 2.5 tissue eq./25 μl (Fig. 5A), this represents less than 1/10th of the activity in whole eggs based on an ED_{50} of 0.004 mg egg eq./μl for NJ eggs containing mature embryos [20] and weighing 700 μg each. Presumably, some of the ecdysiotropic activity in the head region of the *L. dispar* pharate larva resides in the brain, and this activity increases considerably following diapause-break and the reinitiation of development prior to hatch (Table 1).

In *B. mori*, Fugo et al. [17] showed that measurable large and small PTTH-like activities exist not only in whole eggs with mature embryos, but that small PTTH-like activity also exists beginning at oviposition. Furthermore, Fugo et al. [29] showed small PTTH-like activity in the maturing *B. mori* ovary. These studies suggest novel roles for PTTH-like molecules in embryogenesis and ovarian maturation. Only further studies will localize these factors to specific regions of the ovary and embryonated egg and lead to a determination of their roles in the early stages of insect development.

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