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SHORT COMMUNICATION

EVIDENCE FOR A BRAIN FACTOR THAT STIMULATES DEPOSITION OF PUPARIAL HYDROCARBONS IN DIAPAUSING FLESH FLIES

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Puparia from diapausing pupae of the flesh fly *Sarcophaga crassipalpis* are lined with twice as much hydrocarbon as puparia from nondiapausing pupae (J. A. Yoder, D. L. Denlinger, M. W. Dennis and P. E. Kolattukudy, unpublished observations). The additional hydrocarbon favors water conservation during diapause by reducing water loss, enhancing water vapor absorptivity and elevating the critical transition temperature (Yoder and Denlinger, 1991). In this study we seek to identify the source of a hormonal modulator that would account for this difference in hydrocarbons. Our results suggest that a factor unique to the brains of diapause-programmed larvae is responsible for increasing the quantity of hydrocarbon deposited on the puparium. Cyclic AMP elicits the same effect, suggesting that the factor is probably a neuropeptide or another modulator that uses cyclic AMP as a second messenger.

A colony of the flesh fly *Sarcophaga crassipalpis* Macquart was maintained in the laboratory as previously described (Denlinger, 1972). Parental adults were reared under nondiapausing (L:D 15 h:9 h, 25°C) or diapausing (L:D 12 h:12 h, 25°C) conditions. All larvae and pupae were maintained at L:D 12 h:12 h and 20°C. Under these conditions, a high incidence of pupal diapause (>95 %) was observed in the progeny of short-day mothers, while none of the progeny from long-day mothers entered diapause. The photosensitive stage is actually the embryo that develops within the uterus of the female (Denlinger, 1971). Thus, diapausing and nondiapausing groups in this experiment differed only in maternal photoperiod.

Brain extracts, hemolymph and known hormonal agents were tested for their efficacy in stimulating hydrocarbon deposition. Methoprene (Zoecon Corp., Palo Alto, CA), a juvenile hormone (JH) analog, was diluted in acetone and applied topically to larvae. 20-Hydroxyecdysone (Sigma, St Louis, MO) was diluted in 10 % ethanol and injected using a finely drawn glass capillary, and the dibutyryl

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derivative of cyclic AMP (N^6,2′-*O*-dibutyryladenosine-3′:5′-cyclic monophosphoric acid, sodium salt, Sigma; dbcAMP) was co-injected with the phosphodiesterase inhibitor aminophylline (Sigma). To collect larval hemolymph, third-instar larvae were punctured and hemolymph was collected on chilled Parafilm. Hemolymph was withdrawn from pupal heads after first centrifuging pupae in a head-up position. Within a few minutes of collection, the hemolymph was injected into recipient larvae. Brain extracts were prepared from larvae that were within 12 h of pupariation. Brains dissected from chilled larvae were placed in acetone, homogenized and centrifuged; the supernatant was dried under nitrogen and reconstituted in distilled water for injection.

Unless otherwise noted, all recipients were nondiapause-destined larvae that were within 24 h of pupariation. Recipient larvae were immobilized on ice before injection and were returned to ice for a few minutes of recovery after injection. All recipients were returned to 20°C (L:D 12 h:12 h) until pupation (3 days after pupariation). At pupation, a subset of pupae from each experimental group was transferred to 0 % relative humidity (RH) to determine net transpiration rates; the others were permitted to complete adult eclosion and their empty puparia were then analyzed for hydrocarbon content.

To determine net transpiration rates (integumental and respiratory water loss), pupae were held at 0 % RH (generated by anhydrous $CaSO_4$) in sealed glass desiccators. Pupae were first predesiccated [0 % RH, 20°C for 24 h, so that mass change reflects water flux (Wharton, 1985)], and weighed on an electrobalance (Cahn 25, Ventron Co.). After predesiccation, pupae were maintained at 0 % RH and reweighed every 24 h for 5 days. Pupal dry mass was determined after drying the pupae over anhydrous $CaSO_4$ at 50°C for 7 days. Percentage body water content was determined by dividing the water mass (m, the difference between wet and dry mass) by initial wet mass×100 % (Wharton, 1985). In dry air, the pupa's water mass depleted exponentially. The slope of an exponential regression through a plot of $\ln m_t/m_0$ [water masses at time t (m_t) and initial mass (m_0)] *versus* time is the rate of net water loss ($-k_t$) and is expressed as % h^{-1} (Wharton, 1985). Slopes of regression lines were compared according to Sokal and Rohlf's (1984) test for the equality of several slopes.

To quantify the hydrocarbons, empty puparia were extracted with chloroform:methanol (2:1 v/v) for 10 min, dried (N_2) to a volume of 50 μl and passed through a silica gel column (Waters Associates, Milford, MA). Hydrocarbons were eluted with HPLC grade hexane, dried under nitrogen onto predesiccated (0 % RH, 25°C, 24 h), preweighed aluminum pans and held at 0 % RH and 25°C for at least 24 h. Total hydrocarbon quantity was calculated as described by Bligh and Dyer (1959).

Pupal wet mass and percentage body water were not significantly different among the pupae tested (data not shown). In all cases, body water mass was a positive correlate of dry mass ($r>0.89$) and slopes of regression lines describing this relationship were all significantly different from zero ($F>524.27$, d.f.=399, $P<0.001$). Mean wet masses for experimental pupae ranged from 120.51 to

123.98 mg, and body water ranged from 66.28 to 67.31 %; differences between groups were not significant (ANOVA, $P>0.05$). Thus, the total exchangeable water pool for all experimental and control pupae was the same. Percentage body water data were arcsine-transformed prior to statistical analysis.

Puparia from diapausing pupae have twice as much hydrocarbon as their nondiapausing counterparts and the greater amount of hydrocarbon correlates with lower rates of net transpiration for diapausing pupae (J. A. Yoder, D. L. Denlinger, M. W. Dennis and P. E. Kolattukudy, unpublished results; Yoder and Denlinger, 1991; Table 1). The concentration ranges of juvenile hormone analog and 20-hydroxyecdysone that were tested did not affect the quantity of puparial hydrocarbon deposited and water loss from these pupae was similar to that of the controls (Table 1). Armold and Regnier (1975) reported ecdysteroid stimulation of hydrocarbon biosynthesis at pupariation in a closely related species, *Sarcophaga bullata*. But ecdysteroid titers at the time of pupariation are similar for both nondiapause- and diapause-destined larvae of *S. crassipalpis* (Denlinger, 1985); thus, it seems unlikely that ecdysteroids could be responsible for doubling the quantity of puparial hydrocarbon associated with diapause. Unique cycles of JH activity are associated with pupal diapause in flesh flies (Denlinger *et al.* 1984), and this could potentially be a stimulant of hydrocarbon synthesis, but our results do not suggest that JH is involved.

Dibutyryl cyclic AMP, however, when injected with aminophylline, was effective in increasing hydrocarbon levels of the puparium and such pupae lost water at a significantly slower rate than control pupae (Table 1). The accumulation of lipid stores is a feature of the diapause syndrome in *Sarcophaga crassipalpis* (Adedokun and Denlinger, 1985), but dibutyryl cyclic AMP did not increase overall levels of lipid (control=0.073 ± 0.012 mg pupa^{-1}, dbcAMP=0.076 ± 0.014 mg pupa^{-1}), suggesting that cyclic AMP is selective in stimulating hydrocarbon production. Though dibutyryl cyclic AMP promoted the hydrocarbon deposition characteristic of diapause, the pupae themselves did not enter diapause. The efficacy of cyclic AMP in eliciting hydrocarbon deposition is probably limited to a brief interval before pupariation. Elevation of cyclic AMP level after pupariation elicits a very different effect: an injection of cholera toxin, an adenylate cyclase activator that sustains a high level of cyclic AMP for many days after pupariation (Gnagey and Denlinger, 1983), will cause a fly programmed for pupal diapause to reverse the decision and proceed immediately with adult development (Denlinger, 1976). In contrast, our present results suggest that a transient rise in cyclic AMP concentration prior to pupariation may be an essential component of the diapause program.

Recipients of hemolymph from diapause-destined larvae and young (2-day-old) and older (>20 day) diapausing pupae lost water at the same rate as nondiapausing pupal controls (Table 1), suggesting that hydrocarbon levels were not affected by hemolymph transfusions. Similar transfusions into nondiapausing larvae at the time of pupariation also showed no effect.

Recipients of brain extracts from diapause-programmed larvae (7 brain equival-

Table 1. *A comparison of net transpiration rate ($-k_t$) at 20°C and quantities of hydrocarbon (μg per empty puparium) for pupae of* Sarcophaga crassipalpis

Experimental treatment	$-k_t$ (20°C) (% h^{-1})	Hydrocarbon (μg per empty puparium)
Untreated controls		
Nondiapausing pupae	0.024[a]	8.07±0.18[a]
Diapausing pupae	0.009[b]	15.12±0.22[b]
Hormonal agents		
Juvenile hormone analog		
Acetone (5 μl)	0.025[a]	8.08±0.26[a]
0.001 μg	0.024[a]	8.02±0.21[a]
0.01 μg	0.026[a]	7.96±0.24[a]
0.1 μg	0.024[a]	8.06±0.25[a]
1.0 μg	0.025[a]	8.04±0.23[a]
20-Hydroxyecdysone		
10% ethanol (5 μl)	0.023[a]	7.99±0.26[a]
0.001 μg	0.026[a]	8.09±0.25[a]
0.01 μg	0.025[a]	7.91±0.23[a]
0.1 μg	0.024[a]	8.11±0.21[a]
1.0 μg	0.023[a]	8.07±0.19[a]
Cyclic AMP (dibutyryl derivative)		
Water (5 μl)	0.023[a]	8.10±0.21[a]
Aminophylline (10 μg)	0.026[a]	8.13±0.18[a]
dbcAMP (100 μg)+aminophylline (10 μg)	0.018[c]	13.64±0.25[c]
Hemolymph transfusions (50 μl)		
From diapause-destined larvae	0.024[a]	–
From 2-day-old diapausing pupae	0.026[a]	–
From >20-day-old diapausing pupae	0.023[a]	–
Brain extracts		
From nondiapause-destined larvae		
7 brain equivalents	0.025[a]	7.95±0.26[a]
From diapause-destined larvae		
3 brain equivalents	0.025[a]	7.92±0.27[a]
7 brain equivalents	0.016[c]	12.84±0.24[c]

All treated flies were programmed for nondiapause development.

Transpiration rates were calculated from groups of 45 pupae and hydrocarbon content from three replicates of 15 puparia each (mean±S.D.).

Numbers followed by the same letter within a column are not significantly different (ANOVA, $P>0.05$).

ents per larva) had a reduced rate of pupal net transpiration, and this was associated with a higher quantity of puparial hydrocarbon (Table 1). Injection of 3 brain equivalents per larva had no effect. As with cyclic AMP, the brain extract did not alter the pupa's developmental (nondiapause) status. Injection of brain

extracts from nondiapause-programmed larvae neither enhanced quantities of puparial hydrocarbon nor lowered net transpiration rate (Table 1). Thus, a factor unique to a diapause-programmed larval brain, and not simply the presence of additional brain material, was responsible for stimulating hydrocarbon deposition.

While hydrocarbon quantities in normal puparia from diapausing pupae and in hydrocarbon-enhanced puparia from nondiapausing pupae were nearly the same, the net transpiration rates were lower in diapausing pupae. This difference is due to the fact that diapause greatly suppresses the metabolic rate, a feature that also contributes to a low net transpiration rate by reducing respiratory water loss. The difference we note in net transpiration rates between normal nondiapausing pupae and those with enhanced levels of puparial hydrocarbon can probably be attributed directly to the amount of hydrocarbon in the two types of puparia.

These results suggest that the extra puparial hydrocarbon associated with the diapause of *Sarcophaga crassipalpis* is linked to a factor present in the brain of diapause-programmed flies, and the observation that cyclic AMP, a well-known second messenger, mimics this effect indicates that this factor is probably a neuropeptide or another cyclic-AMP-dependent modulator. The chemical identity of the factor remains to be determined, and the pathway leading to the response has not yet been examined.

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