Comparison of 10 days of high E/P ratio to 10 days with low E/P ratio

Patient	Severity of fits		Number of days free from more severe fits	
	Follicular phase	Luteal phase	Follicular phase	Luteal phase
GA	99	46	1	2
EL	125	14	6	9
IW I	58	39	3	7
IW II	56	16	5	10
BA	34.5	1.5	8	10
RO	70	24	5	10
Mean ± SE	73.8 ± 13.4	23.4 ± 6.8	4.7 ± 1.3	8.0 ± 1.0
Student's paired *t*-test	$p < 0.01$		$p < 0.005$	

[4] D. E. WOOLLEY and P. S. TIMIRAS, Endocrinology *70*, 196 (1962).
[5] J. LOGOTHETIS, R. HARMER, F. MORRELL and F. TORRES, Neurology *9*, 352 (1959).

$p < 0.005$; $r = 0.48$, $p < 0.05$; $r = 0.69$, $p < 0.005$; $r = 0.76$, $p < 0.005$). There was a corresponding correlation to oestrogen levels $r = 0.53$, $p < 0.025$; $r = 0.67$, $p < 0.001$; $r = 0.86$, $p < 0.0005$) in the anovulatory cases. A comparison between 10 days with high oestrogen/progesterone (E/P) ratios during the follicular phase, and low oestrogen/progesterone ratios during the luteal phase showed that the severity of fits was significantly higher during the follicular phase than during the luteal phase (see Table) ($p < 0.01$). Correspondingly, a clearly significant lower frequency of days free from more severe fits (secondary generalized convulsions in EL, IW, BA and RO. GA loses her muscular tension and falls down) was observed during the follicular phase ($p < 0.005$) (see Table). This is in agreement with LAIDLOW's[3] demonstration that fits are mildest during the luteal phase, and the oestrogen effect of a lowered electroshock threshold in rats[4] and the induction of Grand Mal by treatment of epileptic patients with oestrogen[5].

Juvenile Hormone Titers in Penultimate and Last Instar Larvae of *Pieris brassicae* and *Barathra brassicae*, in Relation to the Effect of Juvenoid Application

L. VARJAS[1], P. PAGUIA[2] and J. DE WILDE[3]

Research Institute for Plant Protection, Pf. 102, H–1525 Budapest (Hungary); International Rice Research Institute, Los Bagnos (Philippines), and Department of Entomology, Agricultural University, Binnenhaven 7, Wageningen (The Netherlands), 27 August 1975.

Summary. JH titer was determined in the haemolymph of penultimate and last instar larvae of *Pieris brassicae* L. and *Barathra brassicae* L. The differences we observed were consistent with physiological differences between the two species.

Differentiation processes resulting in the metamorphosis of insects are commonly considered as direct consequences of a lowered juvenile hormone (JH) titer. Data on JH titers are surprizingly scarce, despite their importance for the understanding of physiological processes[4,5]. Until recently, appropriate chemical micro-analytical methods were not available and JH titers were determined by means of bioassay and expressed in terms of JH activity per unit volume. Even at present when gaschromatography, high pressure liquid chromatography and radioimmunoassay offer new and more selective methods for titer determinations, a relatively simple extraction procedure coupled with a highly sensitive bioassay cannot yet be considered an obsolete method. We admit that a JH titer determined in samples from the total blood may represent merely an average value and the effective titers near the target tissues probably differ from it to a certain extent. Nevertheless, the actual titer changes in the course of insect development are presumably of much greater extent than such titer variations within the insect body.

In the present investigations we have determined JH titers in penultimate and last instar larvae of two lepidopterous species exhibiting different sensitivities to JH analogues.

Material and methods. Larvae of the cabbage white butterfly, *Pieris brassicae* L. were fed on cabbage plants, those of the cabbage army worm, *Barathra brassicae* L. (= *Mamestra brassicae*) on an artificial medium originally for *Ostrinia nubilalis*[6]. Both species were reared at 25 °C under long-day conditions (18/6 h L/D). Under these circumstances, in *Pieris* the length of the 4th instar (incl. ecdysis) amounted to 40–46 h, that of the 5th (last) instar to 4.5–4.8 days. In *Barathra* the 5th larval instar lasted 72–80 h (incl. ecdysis), the 6th (last) instar 8.5–9 days.

Haemolymph samples of 150–450 µl volume were collected from CO_2-narcotized larvae of known age by clipping off one of the first abdominal prolegs with fine scissors. According to the size of the animals, each sample was derived from different numbers of caterpillars which were sampled simultaneously: 3–20 specimens in *Pieris*, 3–12 specimens in *Barathra*. In different age groups, 2–10 parallel haemolymph samples were taken. The extraction procedure, as well as the application of the *Galleria*-assay in examining the samples, was described earlier in detail[7,8]. Some minor modifications in methods were introduced.

JH titers were expressed in GU/ml haemolymph. One *Galleria* Unit (GU) indicates the amount of juvenile hormone activity (contained in 1 mg of the olive oil-paraffin wax mixture) applied in each test which causes

[1] Research Institute for Plant Protection, Pf. 102, H–1525 Budapest, Hungary.
[2] International Rice Research Institute, Los Bagnos, Philippines.
[3] The authors feel indebted to Prof. V. J. BROOKES for useful comments.
[4] J. DE WILDE, C. A. D. DE KORT and A. DE LOOF, Mitt. schweiz. ent. Ges. *44*, 79 (1971).
[5] K. J. JUDY, M. S. HALL and J. W. MARTIN, unpublished observations (1973), (cit. WEIRICH et al. 1973).
[6] B. NAGY, Acta phytopath. *5*, 73 (1970).
[7] J. DE WILDE, G. B. STAAL, C. A. D. DE KORT, A. DE LOOF and G. BAARD, Proc. kon. ned. Akad. Wet. Ser. C *71*, 321 (1968).
[8] A. DE LOOF and M. VAN DE VEIRE, Experientia *28*, 366 (1972).

positive responses in 50% of test pupae. In our assays 1 GU proved to be equal to the JH activity of 3×10^{-13} g (0.3 pg) DL-*trans, trans, cis* C_{18} *Cecropia* JH)JH-1). If undiluted samples induced positive responses in 20 to 40% of the test animals, the corresponding titers were denoted as 'JH-traces'. The 0 to 20% positive responses, some of them possibly showing only an 'endocrine noise', were denoted as 'no JH'. 8–10 pupae per assay were routinely used.

Results. JH titer values in penultimate and last instar larvae of *P. brassicae* and *B. brassicae* are demonstrated in Figures 1 and 2, respectively. In penultimate instar caterpillars relatively high juvenile hormone titers were generally found, approx. 700–3000 GU/ml in *Pieris*, 3000 to 27000 GU/ml in *Barathra*. In *P. brassicae*, where more determinations were performed, a high level of JH activity seems to be continuously present in the haemolymph of 4th instar larvae. Our results also show that at the time of ecdysis and in freshly moulted last instar larvae, especially in *B. brassicae*, juvenile hormone still circulates in the blood in significant concentrations. Thereafter a more or less rapid decrease in JH titer was observed in both species. In *Barathra* larvae, the JH titer still remained around 70 GU/ml, a low but detectable value, 1 day after the last larval moult. The lowest level of juvenile hormone titer was reached on the 2nd and 3rd day (*Pieris*), or 4th day (*Barathra*) of the last instar. Following this period, when larvae cease feeding and start to search for pupation sites, a relatively prompt increase in JH titer was detected. In *Pieris brassicae* this JH peak seems to be rather low (70–110 GU/ml) and of short duration, being limited only to a period when caterpillars spin their 'girdle' for pupation. In the haemolymph of *Barathra* larvae and prepupae, however, the JH titer apparently remained around 60–120 GU/ml for a longer period, including the beginning of pupal development.

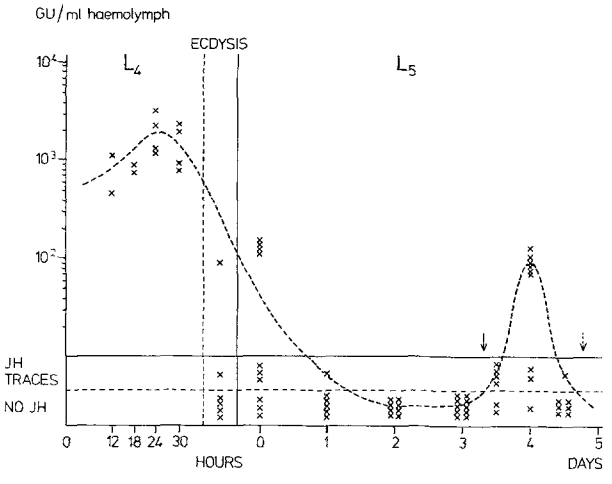

Fig. 1. Juvenile hormone titers in penultimate and last instar larvae of *Pieris brassicae* L. (25 °C, 18/6 h L/D). Arrows: ↓, cessation of food uptake; ↓, time of moult to pupa. Vertical lines: - - - -, cessation of food intake; ——, shedding of cuticle.

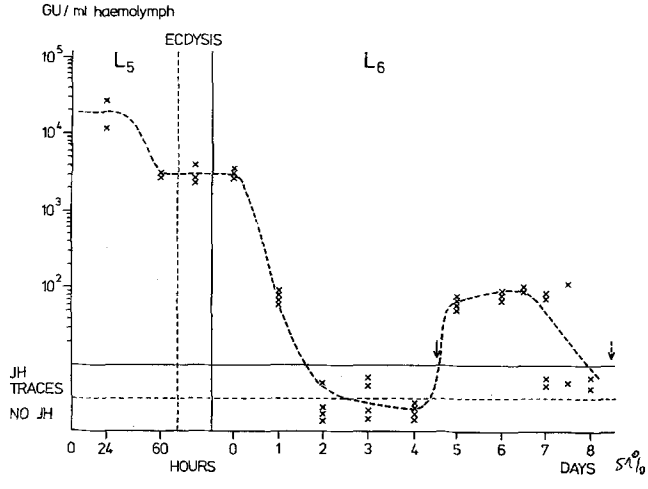

Fig. 2. Juvenile hormone titers in penultimate and last instar larvae of *Barathra brassicae* L. (25 °C, 18/6 h L/D). Arrows: ↓, time of ceasing of food uptake; ↓, time of moult to pupa.

As to JH titers in penultimate instar larvae, our data are rather few to draw more detailed conclusions. In general, JH titer values in these larvae are 10–100 times higher than any titers found in last instar caterpillars. These results do not preclude the existence of a period with lower JH titer. At least in *Pieris*, however, such a period could only be of very short duration.

Discussion. The presence of JH in the haemolymph of moulting larvae is not surprizing and is in good agreement with some data in the literature[9,10]. Significant JH activities were also found in the blood of *Philosamia* and *Manduca* larvae in the beginning of the last instar[4,5]. In *Bombyx* the corpora allata proved to be active in this period[11].

This agrees well with the theory, originated by PIEPHO[12] and referred to by WILLIAMS[13] and many others thereafter, which emphasizes the significance of a low but definite JH titer for larval pupal transformation in Holometabola. The decreasing course of the JH titer in the first half of the last instar coincides with changes in JH sensitivity established by the first author in both species[14,15]. The highest sensitivity to juvenoids with respect to the morphogenetic effect was found on day 3 in *P. brassicae* (at 25 °C) and on day 4 in *B. brassicae* (at 23 °C).

Morphogenetic disturbances (larval-pupal intermediates) could only be evoked in *Barathra* with 100–400 times higher dosages of a juvenoid than in *Pieris* (10 to 400 µg, resp. 0.1–1 µg of ZR-512)[14,15]. In freshly moulted last instar larvae (on day 0), we found an approx. 30-fold difference in JH titer which might occur also on subsequent days. The difference in JH sensitivity between the two species is to a certain degree reflected in the corresponding juvenile hormone titers. Nevertheless, we must be very cautions in drawing too definite conclusions in the absence of information on the chemical nature of the natural juvenile hormone(s) of these Lepidoptera[16].

The re-appearance of JH near the middle of the last instar in larval haemolymph points to a renewed activity of corpora allata in this period. KAISER[17] demonstrated a corresponding phase in the secretion of these endocrine glands in *P. brassicae* by means of histological methods. Some other investigations also revealed a similar period

[9] J. M. LEGAY, C. r. Séanc. Soc. Biol., Paris, D *144*, 512 (1950).
[10] M. REHM, Roux' Arch. EntwMech. Org. *145*, 205 (1951).
[11] S. FUKUDA, J. Fac. Sci., Tokyo Univ. Sec. IV *6*, 477 (1944).
[12] H. PIEPHO, Verh. dt. zool. Ges., 62 (1951).
[13] C. M. WILLIAMS, Biol. Bull. *121*, 572 (1961).
[14] L. VARJAS, Gen. comp. Endocr. *22*, 414 (1974).
[15] L. VARJAS and M. TÓTH, unpublished observations (1975).
[16] G. BENZ, Experientia *29*, 1437 (1973).
[17] P. KAISER, Roux' Arch. EntwMech. Org. *144*, 99 (1949).

with higher corpus allatum activity in the last instar larvae of *Melanoplus*, *Pyrrhocoris*, *Anisolabis*, and *Locusta*[18–21].

The periodic functioning of the corpus allata seems, therefore, to continue in last instar larvae, although perhaps at a reduced rate. The role of fluctuations in the rate of enzymatic breakdown of JH with respect to the changes in JH titer has still to be determined in both *Pieris* and *Barathra*.

The morphogenetic and physiological significance of higher JH titers in the second half of the last larval instar is as yet not clear. In *Pieris* and *Barathra*, possibly also in other Holometabola, it occurs at a time when the critical period for the determination of metamorphosis is over and the larvae prepare themselves for pupation (e.g. spinning of webs or cocoons). The presence of JH may be related to its inhibitory effect on growth and differentiation of imaginal discs and other developing structures. Their precocious differentiation would be unfavourable for the insect until an appropriate environment for pupation has been ensured. In this connection it is very suggestive to compare our idea with a few data from the literature referring to a significant corpus allatum activity during larval diapause in *Chilo*, *Plodia*, and *Diatraea*[22–24]. It seems that in larval diapause the JH peak, typical also for non-diapause development, is temporarily stabilized. It is also worthwhile mentioning that in *Pieris brassicae* the JH peak found in the last instar coincides with the critical period of light sensitivity for pupal morphological colour change[25]. This peak cannot bear a relation to 'girdle' spinning behaviour itself, as, according to BENZ[16], the endocrine induction of this behaviour occurs within the first 60 hours of the instar.

In conclusion we can state that in *Pieris* and *Barathra*, the complex processes leading to metamorphosis are accompanied by subtly timed changes in JH titer, which may partly explain the species specific variations found in morphogenetic and physiological responses to exogenous hormone supply.

[18] M. V. MENDES, Biol. Bull. *94*, 194 (1948).
[19] V. J. A. NOVÁK and E. CERVENKOVÁ, Acta Symp. Evol. Insects, Prague (1959), p. 152.
[20] K. OZEKI, Sci. Papers Coll. gen. Educ. Univ. Tokyo *15*, 149 (1965).
[21] P. JOLY, Ann. Soc. ent. Fr. *3*, 601 (1967).
[22] M. FUKAYA and J. MITSUHASHI, Jap. J. appl. Ent. Zool. *2*, 223 (1958).
[23] Y. WAKU, Sci. Rep. Tohoku Univ. Biol. *26*, 327 (1960).
[24] C.-M. YIN and G. M. CHIPPENDALE, J. Insect Physiol. *19*, 2403 (1973).
[25] A. OLTMER, Roux' Arch. EntwMech. Org. *160*, 401 (1968).

Monolayer Cultures of Normal Adult Rat Adrenocortical Cells: Steroidogenic Responses to Nucleotides, Bacterial Toxins and Antimicrotubular Agents

M. J. O'HARE[1]

Division of Pathology, Chester Beatty Research Institute, Fulham Road, London, SW3 6JB (England), 22 August 1975.

Summary. Monolayer cultures of normal rat adrenocortical cells were treated with agents which stimulate steroidogenesis by Y-1 adrenal tumour cells. Choleragen was active, whereas cyclic nucleotides other than cyclic AMP, bacterial endotoxins and antimicrotubular agents were inactive.

Normal adult rat adrenocortical cells can be maintained in vitro for several months as primary monolayer cultures, while retaining their morphological and steroidogenic responses to ACTH and cyclic AMP[2–4]. Cultures of differentiated adrenal tumour cells, particularly the Y-1 established cell-line[5], have also been extensively used in studies of adrenal-specific functions[6]. These tumour cells, however, respond to a number of substances other than ACTH and cyclic AMP. They are stimulated by other cyclic nucleotides such as cyclic CMP[7], by bacterial toxins including choleragen (*V. cholerae* enterotoxin) and unpurified endotoxins[8,9] and by microtubule-disrupting agents such as colchicine[10,11].

The long-term responses of cultured normal adrenal cells to these agents have therefore been examined, because the validity of tumour cells as models of adrenal function evidently depends on the extent to which they have retained normal responses.

Materials and methods. Tissue culture. Confluent monolayer cultures of approximately $0.5–1.0 \times 10^6$ cortical cells were prepared from the zona fasciculata-reticularis of adrenal glands from 8-week-old (200 g) male Wistar rats, using the collagenase-hyaluronidase disaggregation procedure described previously[2]. Cultures were maintained at 37 °C in 25 cm² polystyrene culture flasks (Falcon) with 5 ml Dulbecco's Eagle's medium containing 15% fetal calf serum (GIBCO), plus 100 µg/ml each of penicilllin and streptomycin with a gas-phase of 10% CO_2 in air. Culture medium was changed every 24 h and retained for measurement of steroid content, and the number of cortical cells in each culture was determined by direct counting of cells in randomly-chosen fields of known area under an inverted phase-contrast microscope.

Cyclic nucleotides, analogs thereof, nucleotides, nucleosides and purine and pyrimidine bases (Sigma Chemical Co.) were added directly to the cultures dis-

[1] This work was supported by the Medical Research Council of Great Britain (grant No. 970/656/B). I am grateful to Miss R. MAGEE for technical assistance and Prof. A. M. NEVILLE for continued support.
[2] M. J. O'HARE and A. M. NEVILLE, J. Endocr. *56*, 529 (1973).
[3] M. J. O'HARE and A. M. NEVILLE, J. Endocr. *56*, 537 (1973).
[4] M. J. O'HARE and A. M. NEVILLE, J. Endocr. *58*, 447 (1973).
[5] V. BUONASSISI, G. SATO and A. I. COHEN, Proc. natn. Acad. Sci., USA *48*, 1184 (1962).
[6] J. KOWAL, Recent Progr. Horm. Res. *26*, 623 (1970).
[7] J. KOWAL, Endocrinology *93*, 461 (1973).
[8] S. T. DONTA, M. KING and K. SLOPER, Nature, Lond. *217*, 246 (1973).
[9] J. WOLFF, R. TEMPLE and G. H. COOK, Proc. natn. Acad. Sci., USA *70*, 2741 (1973).
[10] R. TEMPLE, J. A. WILLIAMS, J. F. WILBER and J. WOLFF, Biochem. biophys. Res. Commun. *46*, 1454 (1972).
[11] R. TEMPLE and J. WOLFF, J. biol. Chem. *248*, 2691 (1973).