4. Mortier, W. J., *Struct. Bonding*, 1987, **66**, 126–144.
5. Dutt, N. V. K., Kulkarni, S. J. and Ravikumar, Y. V. L., *Zeolites*, 1995, **15**, 373–376.
6. Hati, S. and Datta, D., *J. Phys. Chem.*, 1994, **98**, 10451–10454.
7. Hati, S. and Datta, D., *J. Phys. Chem.*, 1995, **99**, 10742–10746.
8. *Refractometry and Chemical Structure*, S. S. Batsavon, Consultants Bureau, 1961, p. 38.
9. Komorowski, L., *Struct. Bonding*, 1993, **80**, 46–70.
10. Dutt, N. V. K., Kulkarni, S. J. and Ravikumar, Y. V. L., unpublished results.

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Azadirachtin-induced changes in ecdysteroid titres of *Spodoptera litura* (Fabr.)

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Using radioimmunoassay, insect moult hormone (20-hydroxyecdysone) level was monitored in azadirachtin-injected and control larval (final instar) insects of *Spodoptera litura* (Fabr.). Azadirachtin has significantly reduced the hormone level, which suggests its interference in hormonal regulation, growth and development of the insects.

AZADIRACHTIN, a tetranortriterpenoid, has a number of isomers, of which azadirachtin A contributes the bulk. It has varied effects, viz. antifeedant, repellent, growth regulatory, ovicidal and insecticidal properties against many insects[1–6].

Studies on physiological effects of azadirachtin showed that it affects the hormonal titre within the insect through tropic factors. The developmental effects of azadirachtin are attributed to a disruption of endocrine events since the azadirachtin molecule itself has a unique resemblance of the major insect steroid hormone, ecdysone[7]. Complete moult inhibition is shown to be due to either a total blockage of haemolymph ecdysteroid or to a delay in the appearance of the last ecdysteroid peak with or without a reduction in peak height and a slow abnormal decline in the peak[8–11] or the action of azadirachtin as anti-ecdysteroid by blocking the ecdysone-binding sites[12,13]. Ecdysone production of prothoracic glands incubated in the presence or absence of azadirachtin and/or prothoracic tropic hormone (PTTH) have shown that azadirachtin does not act directly on the prothoracic glands in *Calliphora vicini*, *Bombyx mori* and *Heliothis virescence*[14,15]. Further studies showed that azadirachtin blocks the release of neurosecretory material from the corpora cardiaca with a reduced turnover seen as a subsequent accumulation of material within the system[16]. Detailed studies on single injected dose of azadirachtin (1 µg/g body weight) into final instar larvae of *Spodoptera litura* have shown deleterious effect on food utilization, midgut enzymes[17,18], haemolymph constituents[19], corpora allata volume, median neurosecretion and carry over effects on ovarian development[20].

In the moulting process of insects, ecdysteroids [ecdysone and 20-hydroxyecdysone (ecdysterone)] play a vital role. Ecdysterone titre increased with the age of the larvae. Since the azadirachtin treatment affects the growth and development of the insects, it was interesting to know the effect of the same on those important hormones. In this communication, results on the azadirachtin-induced changes on ecdysterone titres of final instar *S. litura* are discussed.

The haemolymph ecdysteroid titres were determined by RIA basically following Borst and O'Connor[21], Chang and O'Connor[22]. Ecdysteroid antibody A and azadirachtin A were gifts from Prof. E. Chang, California and Prof. H. Rembold, Max Planck Institute for Biochemistry, Munchen, Germany, respectively. Radiolabelled ligand [(^{3}H)ecdysone (50 µCi mmol^{-1})] was purchased from NEN (USA) and scintillation cocktail (Riatron) was from Kontron (Switzerland).

All other reagents [boric acid, bovine serum albumin (BSA) fraction V, sodium chloride, ammonium sulphate and methanol of extra pure quality] were purchased from SISCO Research Laboratory (India).

Azadirachtin was dissolved in 70% ethanol [(ethanol : water) (70 : 30 v/v)] and injected (@ 1 µg/g body weight) into the newly moulted VI instar larvae of *S. litura*, at the base of one of the I pair of prolegs, using Hamilton^R syringe. Control larvae were injected with 70% ethanol only. The treatments were replicated five times and all the experimental insects were fed with semi-synthetic diet.

Haemolymph samples (40 µl) were drawn at 24 h interval from azadirachtin-treated and control larvae. Samples (40 µl) were collected in clean sample vials and ecdysteroids extracted with 70% methanol : water (70 : 30 v/v). The mixture was kept at 4°C for overnight to facilitate precipitation and centrifuged at 2,000 *g* for 10 min in a table top centrifuge. The supernatant was dried in the counting vials at 36°C.

The schematic diagram for the assay is given in Figure 1. Throughout the assay, RIA buffer [containing borate buffer (100 mM; pH 8.5), BSA (0.1%), sodium azide (0.1%) and sodium chloride (0.5%)] was used, unless otherwise stated. The antiserum was diluted in antibody dilution buffer [same as that of RIA buffer except for the high BSA concentration (5%)]. Radiolabelled ligand was diluted in RIA buffer to get 50% antibody binding and 18,000 dpm radioactivity.

The binding of standard or unknown sample (B) was

Radiolabllled ecdysone [(^{3}H) ecdysone] 100 µl
↓
Antiserum 'A' 100 µl + Evaporated sample (or) 20 µl of cold ecdysteroid standard
↓
Mix well and incubate for 12 h at 4°C
↓
Terminate the reaction by 200 µl of ice cold saturated ammonium sulphate
↓
Mix well and keep at 4°C
↓
Centrifuge at 2000 *g* for 10 min
↓
Discard the supernatant and wash the pellet with 400 µl 50% saturated ammonium sulphate
↓
Centrifuge at 2000 *g* for 10 min
↓
Discard the supernatant and redissolve the pellet in 60 µl of distilled water
↓
Add 1 ml of scintillation cocktail and mix well
↓
Read in liquid scintillation counter.

Figure 1. Protocol for the assay.

Table 1. Effect of azadirachtin on 20-ecdysone (ng ml^{-1}) concentration of haemolymph

Hours after treatment	Control	Treatment
24	117.1 ± 5.4	14.7 ± 3.6
48	230.5 ± 14.0	41.2 ± 4.7
72	267.4 ± 19.3	153.7 ± 26.7
96	533.4 ± 43.4	389.9 ± 9.1
120	273.4 ± 12.7	121.7 ± 4.9
Mean	284.5 ± 18.9	144.2 ± 9.8

Comparison by 2-FCRD.
Values are mean (± SE) of five determinations.
CD between main treatment = 16.23.
CD between subtreatments = 25.67.
CD between main and subtreatment = 36.28.

expressed as a per cent of maximum binding ($B/B_0 \times 100$). The logit-transformed values were plotted against log-ecdysteroid concentrations (cold ecdysteroid standard – ecdysone and 20-hydroxyecdysone). From the standard curve, the concentration of ecdysterone was estimated by interpolating the values. The titre data were expressed in ng ml^{-1} 20-hydroxyecdysone equivalents.

The concentration of the hormone at 24 h after moulting into sixth instar was 117.1 ng ml^{-1} which increased by two folds (230.5 ng ml^{-1}) at 48 h. Azadirachtin treatment dramatically decreased the hormonal level (14.7 ng ml^{-1}) at 24 h. Subsequent increase in the hormonal concentration of the treated larva was similar to that of the normal insects, but with a significantly lower values (Table 1). Some insects could not complete their life cycle and, both larval (8.7%) and pupal (34%) deformities were observed in the treated insects.

A similar situation was also reported[23] in *S. litura*. The difference between the control and treated in hormonal titres may be due to the effect of azadirachtin on ecdysteroid biosynthesis or its metabolism[24].

The haemolymph 20-hydroxyecdysone level remained low during larval feeding and began to increase at the end of the active feeding stage (48 to 72 h) reaching the maximum a day before ecdysis. This is in agreement with the observations on *Plusia aganata*[25]. It was observed[26] that the increase in the haemolymph 20-hydroxyecdysone concentration coincides with the increase in fat body protein level in ligated *S. litura*, suggesting greater synthetic activity of the brain.

The declining hormonal titre in the haemolymph may be due to the greater binding with the peripheral tissues which become physiologically active during this stage, leading to wandering behaviour[27,28].

The 20-hydroxyecdysone levels of the haemolymph of the insects reared on semi-synthetic diet are lower compared to those reared on castor leaf[23] which may be due to excessive moisture in the diet causing greater retention by the haemolymph. But the present values are in general agreement with the observations[26] on *S. litura* maintained on semi-synthetic diet.

1. Pradhan, S., Jotwani, M. G. and Rai, B. K., *Indian Farming*, 1962, **12**, 7–11.
2. Butterworth, J. H. and Morgan, E. D., *J. Insect Physiol.*, 1971, **17**, 969–977.
3. Rembold, H. and Sieber, K. P., in *Natural Pesticides from the Neem Tree (Azadirachta indica A. Juss)* (eds Schmutterer, H., Ascher, K. R. S. and Rembold, H.), GTZ, Eschborn, West Germany, 1981, pp. 75–91.
4. Schmutterer, H., *Annu. Rev. Entomol.*, 1990, **35**, 271–297.
5. Koul, O. and Isman, M. B., *J. Insect Physiol.*, 1991, **37**, 591–598.
6. Shashi Gupta and Rao, P. J., *Shashpa*, 1994, **1**, 39–46.
7. Ruscoe, C. N. E., *Nature*, 1972, **236**, 159–160.
8. Redfern, R. E., Kelly, T. J., Borkovec, B. and Hayes, D. K., *Pestic. Biochem. Physiol.*, 1982, **18**, 351–356.
9. Sieber, K. P. and Rembold, H., *J. Insect Physiol.*, 1983, **29**, 523–527.
10. Schluter, U., Bidmon, H. J. and Grewe, S., *J. Insect Physiol.*, 1988, **31**, 773–777.
11. Mordue (Luntz), A. J., Evans, K. A. and Charlet, M., *Comp. Biochem. Physiol.*, 1986, **C85**, 297–301.
12. Barnby, M. A. and Klocke, J. A., *J. Insect Physiol.*, 1990, **36**, 125–131.
13. Kauser, G. and Koolman, J., in *Advances in Invertebrate Reproduction* (ed. Engels, W.), Elsevier, Amsterdam, 1984, vol. 3, pp. 602.
14. Koolman, J., Bidmon, H. J., Lehmann, M. and Kauser, G., in *Endocrinological Frontiers in Physiological Insect Ecology* (eds Sehnal, F., Zabza, A. and Denlinger, D. L.), Wroclaw Technical Univ. Press, Wroclaw, 1988, vol. 1, pp. 55–67.
15. Bidmon, H. J., Kauser, G., Mobus, P. and Koolman, J., in Proceedings of the 3rd International Neem Conference (eds Schmutterer, H. and Ascher, K. R. S.), GTZ, Eschborn, Germany, 1987, pp. 232–272.

16. Subrahmanyam, B., Muller, T. and Rembold, H., *J. Insect Physiol.*, 1989, **35**, 493–500.
17. Rao, P. J. and Subrahmanyam, B., *J. Entomol. Res.*, 1987, **11**, 166.
18. Ayyangar, G. S. G. and Rao, P. J., *Indian J. Entomol.*, 1989, **51**, 373.
19. Ayyangar, G. S. G. and Rao, P. J., *Indian J. Entomol.*, 1990, **52**, 69.
20. Shashi Gupta and Rao, P. J., *Indian J. Entomol.*, 1990, **52**, 589.
21. Borst, D. W. and O'Connor, J. D., *Science*, 1972, **178**, 418–419.
22. Chang, E. S. and O'Connor, J. D., in *Methods of Hormone Radioimmunoassay* (eds Jaffe, B. M. and Behrman, H. R.), Academic Press, New York, 1977, pp. 797–812.
23. Kranti, K. R., Ph D thesis, P.G. School, IARI, New Delhi, 1991, p. 80.
24. Smith, S. L. and Mitchell, M. J., *Biochem. Biophy. Res. Commun.*, 1988, **154**, 559–563.
25. Zhu, X. X., *Contrib. Shangui Inst. Entomol.*, 1986, **4**, 65–69.
26. Tojo, S., Morito, M., Agni, N. and Hiruma, K., *J. Insect Physiol.*, 1985, **31**, 283–292.
27. Bidmon, H. J., Stumpf, W. E. and Granger, N. A., *Cell Tissue Res.*, 1991, **263**, 183–194.
28. Dominick, O. S. and Truman, J. W., *J. Expt. Biol.*, 1985, **177**, 45–68.

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Isozyme polymorphism in diploid and heat shock-induced tetraploid Indian major carp, *Labeo rohita* (Hamilton)

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Heat shock-induced tetraploids of *Labeo rohita*, an Indian major carp, were studied for esterase, glucose-6-phosphate dehydrogenase (G6PD) and xanthine dehydrogenase polymorphism involving different tissues. Polymorphic loci were identified in eye lens, kidney and skeletal muscle for G6PD and only in kidney for esterase of tetraploids. In general, intensification of isozyme bands was observed in tetraploids. The polymorphism in G6PD-1 and 2 locus in eye lens, G6PD-2 in kidney and G6PD-1 in skeletal muscle and EST-1 locus in kidney could be used as reliable marker in identifying tetraploid stocks from diploids.

Isozymes offer a potentially powerful and reliable tool in resolving genetic relatedness/divergence employing the degree of polymorphism of diverse alleles at different loci involved in translating specific enzymes and their varied multiple molecular forms, i.e. isomers. They have amply been used as molecular tags in genetic, phylogenetic, taxonomic, evolutionary studies and in strain or type identification[1–5].

Enzymes are mostly primary products of transcriptionally active genes and it is assumed that specific enzyme profile is the reflection of the genetic make-up of a given species and may be used as 'finger print' considering all other variables as constant. The present study was aimed at identifying any induced variation due to increased ploidy level (diploid to tetraploid) in three different enzyme profiles across diverse tissues and to assessing the feasibility of employing such altered profile (as marker) in identification of tetraploid fishes. Confirmation of tetraploid status has largely been based on chromosome counting[6], cellular volume measurement[7], DNA content[8] and protein electrophoresis[9].

Ploidy manipulation was found as a novel approach in altering diverse traits in fish system[8,10] and promising results in many cases encouraged us to undertake the same with *Labeo rohita* in the Central Agricultural Research Institute, Port Blair during 1993. Thermal shock-induced tetraploid stocks[11] were developed and reared under in house hatchery and in natural pond condition for evaluation and characterization. Specimens were collected from the polycraft pool and pond after eight months of rearing. After having tetraploid status confirmed (through chromosomal count following standard technique[12]), liver, kidney, eye lens and skeletal muscle were dissected out immediately on sacrificing the experimental fishes. The cell lysate was prepared in tissue homogenizing buffer[13]. Homogenates were loaded and electrophoresed following standard procedure[5] in a 5% native PAGE using discontinuous buffer system. Isozymes were detected through specific histochemical staining following Shaw and Prasad[14] for G6PD and Paul *et al.*[15] for esterase (EST) and xanthine dehydrogenase[10] (XDH). The mobility of individual band was calculated through the measurement of R_m values.

The profiles of all the three isozymes are presented in Figures 1–3 along with corresponding zymograms. The allellic expression of G6PD (E.C.1.1.1.49) could be distinctly grouped into two regions in liver and three regions in kidney of tetraploids whereas only two activity zones were found in diploids (Figure 1). The anodal band (locus-3) was a homozygous locus and represented by a single intensely stained band in tetraploids with slow mobility. In diploid, this locus was represented by light-stained band coupled with faster mobility. To the contrary, the presence of an additional zone of activity (locus-2) in the kidney was found to be unique in tetraploids. In the eye lens, G6PD was expressed in three distinct loci (locus-1, 2, 3) of tetraploids (Figure 1, lanes 10 & 11). Three alleles of heterozygous nature