MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON..... 4 October 2002 .....
.....[redacted].....
Sec. Research Graduate School Committee

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ERRATA

p xiii Appendix 37 remove paragraph indentation
p 3 first para 3rd line: "with a rigid exoskeleton" for "with rigid exoskeleton"
p 3 second para 4th line: "cement layer, and a predominantly" for "cement layer, a predominantly"
p 4 first para 4th line: "trophic factors" for "tropic factors"
p 4 second para 9th line: " folding, leading to " for "folding of the epidermis which lead to"
p 5 second para 2nd line: "Ametabola" for "ametabola" and "Hemimetabola" for " hemimetabola"
p 5 second para 3rd line: "Paurometabola" for "paurometabola" and "Holometabola" for "holometabola"
p 5 third para 5th line: "which are developmentally" for "which developmentally"
p 5 last para last line: "cyclorrhaphous" for "cyclorraphous" and "glands," for "glands:"
p 6 first para 4th line: " by" for "by:" and "ecdysteroids," for "ectdysteroids:"
p 6 first para 5th line: "mitochondria," for "mitochondria:"
p 6 first para 6th line: "channels," for "channels:" and "fragmentation," for "fragmentation:"
p 7 last para 7th line: " part of the ovariole" for " part of ovariole"
p 7 last para 8th line: "into a vitellarium" for "into vitellarium"
p 9 last but one line: "Oocyte resorption" for "Oocytes resorption"
p 13 second para 2nd line: "key hormones" for " a key hormone"
p 14 first para last line: "of the brain" for "of brain"
p 15 first para 5th line: "cyclorrhaphous" for "cyclorraphous"
p 15 last line: "24 h" for "24 hr"
p 18 second para 3rd line: "as the main cellular component, the so-called" for "mostly cellular component, so called"
p 20 last line first para: "*et al.*" for "*et al,*"
p 20 second para first line: "1047.6" for "1,047.6"
p 20 third para 2nd line: "*bullata,*" for "*bullata:*"
p 21 second para 5th line: " Kataoka *et al.* (1989)" for " In 1989, Kataoka *et al.*"
p 27 last para 7th line: "22-phosphate" for "22-phophate"
p 27 last but one line: "between ecdysone" for "between individual ecdysone"
p 30 second para 3rd line: "Lepidoptera" for "*Lepidoptera*"
p 30 second para 6th line: "Diptera" for "*Diptera*"
p 32 line 3: " At the molecular" for " At molecular level"
p 32 last para 8th line: "from the larval to the pupal" for " from larval to pupal"
p 32 last para 11th line: "is brought about" for " is acted"
p 32 last para 13th line: "*Est-JH*" for "Est-JH"
p 32 last para 14th line: "*Ra-JHE* " for "Ra-JHE"
p 32 last but one line: "*Ra-JHE* " for "Ra-JHE"
p 33 first para 2nd line: "*Aedes aegypti*" *for* "*A. aegypti*"
p 33 last but one line: "occurred" for "ocurred"
p 34 second para 3rd line: "essential" for "essensial"
p 35 last para 2nd line: "hormones," for "hormones:"
p 36 first para 2nd line: "myiasis" for "myasis"
p 38 first para 3rd line: "at 27 º" for "of 27 º"
p 39 last but one line: "(between 0500 and 0700 h)" for "(0500 and 0700 h)"
p 41 last para 2nd line: "post-feeding larva (PFL, Fraenkel…" for "PFL (Fraenkel…"
p 42 second para 2nd line: "cyclorrhaphous" for "cyclorraphous"
p 44 second para 2nd line: "because of the small size," for "due to the small size:"

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p 46 first para 2nd line: "time-consuming" for " time consuming"
p 48 3rd last line: "pers.comm." for "pers.Comm."
p 53 19th line: "microlitres" for "microlitre" and "were" for "was"
p 53 last line: "24 h" for "24 hours"
p 64 first para 2nd line: " and ecdysteroids" for " i.e. ecdysteroids"
p 66 first line: " events and ecdysteroid levels" for " events ecdysteroid levels"
p 67 second para 2nd line: "phragmata" for "phragma"
p 72 last but one line: "unlabeled" for "Unlabeled"
p 75 first para 3rd last line: "several replicates showed similar patterns" for "several rearing medium replicates showed similar pattern"
p 76 second para 5th line: "until, approximately in the middle of the next scotophase,..." for "until approximately the middle of the next scotophase..."
p 76 last para first line: " In " for "I "
p 80 3rd last line: "where SEM too small" for "where too small"
p 81 second para first line: "0 h" space for "0h"
p 84 last but one line: "where SEM too small" for "where too small"
p 85 3rd last line: "oviposition" for "ovipositition"
p 86 first para last line: "intervals" for "interval"
p 87 first para 5th line: " higher" for "thicker"
p 100 Fig.32: delete Y axis (vertical) label and read "mean weight, mg"
p 114 first para 3rd last line: "because of" for "due to"
p 114 first para last but one line: comma after "however"
p 114 last but one line: "when SEM too small" for "when too small"
p 120 first para 4th line: " protein" for "ptotein"
p 122 last line: "ecdysteroid" for "ecdystroid"
p 126 first para 2nd line: "aspects which will" for "aspects will"
p 128 last line of first para: "cyclorrhaphous" for "cyclorraphous"
p 129 last para 2nd line: "It has been" for " This has been"
p 130 last para 12th line: "identified to carry out" for "identified as to carry out"
p 130 last para 13th line: " knirps" for "knirp" and "knirps-related" for "knirp-related"
p 130 4th last line: "*kni*" for "kni"
p 130 3rd last line: "*tll*" for "tll"
p 135 first para 3rd line: "and 26 h, prior" for "and 26 h prior"
p 135 first para 13th line: "prothoracic" for "prothiracic"
p 140 first line: "smoothning" for "smoothening"
p 140 first para 4th line: "occurs" for "occurred"
p 140 last para first line: "smoothning" for "smoothening"
p 141 first para 7th and 9th line: "O/on the basis of" for "B/based on"
p 142 first line: "during the prepupal" for "during prepupal"
p 144 first para 7th line: "nuclear" for "nucleic"
p 150 5th line: "carcass" for "carcas"
p 150 first para 14th line: "Anautogenous" for "Being of an anautogenous strain,"
p 153 first para last line: "cyclorrhaphous" for "cyclorraphous"
p 153 last para 6th line: "suggest" for "suggests"
p 156 first line: "amounts" for "amount"
p 158 2nd para 5th line: "bottle at room temperature" for "bottle. at room temperature"
p 159 point 3: " Bouin's" for "Bouins' "
p 159 point 6: "up to" for "upto"
p 242 8th reference 2nd line: "ingestion of components" for "ingestion components"
p 243 2nd reference 5th line: "Developments" for "Development"

p 243 5th reference: after "(1988)" read "Ultrastructural localization of phenoloxidases in cuticle and hemopoietic tissue of the blowfly *Lucilia cuprina*. Tissue Cell 20(3): 405 – 420."
p 244 7th reference 3rd line: " J. Exp. Zool." for "J. Exp. Zoo."
p 245 5th reference 2nd line: "JH" for "jh"
p 245 8th reference first line: "Isolierung" for "isolierung"
p 245 8th reference 2nd line: "Hormone der Insekten" for "hormone der insekten" and "Naturforsch." for "Naturforsh"
p 246 4th reference 2nd line: " Diptera, Drosophilidae" for "diptera, drosophilidae"
p 246 6th reference first line: "Insects" for "Insect"
p 247 5th reference second line: "In: Pest Control and Sustainable Agriculture, S.A Corey, D.J Dall and W.M Milne eds., CSIRO Australia." for " Fifth Australian Entomology Research Conference."
p 247 6th reference first line: "Diptera, Calliphoridae" for " diptera, calliphoridae"
p 247 8th reference 2nd line: "Meig." for "Meig"
p 249 2nd reference 2nd line: "Deutsch." for "Disch."
p 249 last line: "Regulatory Peptides" for "Regul. Pept."
p 251 2nd reference first and 2nd line: "*sericata*" for "*cericata*" and space between "cells.," and "Ann."
p 252 2nd reference 2nd line: "*suppressalis*" for "*suppresalis*"
p 253 last reference first line: " Gilbert" for "Gilber"
p 254 7th reference first line: "O'Connor" for " O' Conor"
p 254 last reference last line: comma after "Degradation"
p 255 first reference 2nd line: "Ecdysone Research" for "ecdysone research"
p 255 7th reference first line: "formation in the embryo" for "Formation in the Embryo"
p 256 5th reference 2nd line: "ecdysteroids" for "evdysteroids"
p 257 4th reference 2nd line: "Molekülstrukturanalyse" for "Molekulstranalyse"
p 257 6th reference 2nd and 3rd line: "phosphates" for "phophates" and "Insect" for "Insect."
p 258 first reference first line: "arthropod" for "Arthropod"
p 258 4th reference 3rd line: "*Tenebrio molitor*" for "tenebrio molitor"
p 259 first reference first and 2nd line: "Biogenese des Ecdysons, I, Umwandlung von Cholesterin in Ecdysons" for "biogenese des ecdysons, I, Umwandlung von cholesterin in ecdysons"
p 259 delete entire 2nd reference (not cited in text)
p 259 3rd reference 2nd line: "Ecdysons" for "Ecdysones"
p 259 4th reference first line: "Kataoka, H." for "Kataoka, ,H"
p 259 6th reference 2nd line: "(1987b), Isolation and primary structure of the eclosion hormone of the tobacco hornworm, *Manduca sexta*." for " (1987b)
p 260 5th reference first line: "(1987), Amino acid sequence of eclosion hormone of the silkworm, *Bombyx mori*." for (1987)
p 261 3rd reference 2nd line: "*Chymomyza costata*" for "chymomyza costata"
p 261 4th reference 2nd line: "hormone" for "bbormone"
p 262 5th reference 2nd line: "quantification of juvenile hormone biosynthesized by larval and adult Australian sheep blowfly" for "Quantification of Juvenile Hormone Biosynthesized by Larval and Adult Australian Sheep Blowfly"
p 263 7th reference 2nd line: "(Diptera, Culicidae), European" for "(diptera, culicidae), Euro."
p 263 8th reference first line: "gnathocephalon" for "gnatocephalon"
p 263 last reference first line: "Insect Life" for "insect life"
p 264 4th reference 2nd line: "gene" for "gen"

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p 266 last reference 2nd line: "*knirps*" for "*knirp*"
p 267 2nd reference first line: "zoologists., Cambridge University Press" for "zoologist., Cambridge university Press"
p 267 4th reference 2nd line: "Insektenmetamorphose. Über" for "Insektenmethamorphose. Uber"
p 267 5th reference 3rd line: "expressed" for "express'"
p 267 7th reference 3rd line: "Meigen" for "meigen"
p 269 2nd reference first, 2nd and 3rd line: " the" after " analysis of" and "maturing" and "*Locusta migratoria*" for "maturating" and " *Periplaneta americana*"
p 269 last reference last line: "Symposium" for "symposium"
p 270 first reference first and 2nd line: "activity of dipteran ring glands and activation by the prothoracicotropic hormone" for "Activity of Dipteran Ring Glands and Activation by the Prothoracicotropic Hormone"
p 270 5th reference 3rd line: " Development" for "Dev."
p 270 first reference first line: "Jäckle" for "Jackle"
p 271 3rd reference first line: "Jäckle" for "Jackle"
p 271 6th reference first line: "Scharrer, B. and Hadorn, E." for "Scharrer, B., Hadorn, E." and "The" for "the"
p 272 first reference first line: "control of rates of metamorphic development in the tobac hornworm" for "Control of Rates of Metamorphic Development in the Tobacco Hornworm"
p 274 6th reference first line: "Hogness" and "Hognes"
p 274 7th reference first line: "Imray, F.P. and Horn" for "Imray, F.P. , Horn"
p 275 first reference 2nd line: "function" for "fuction"
p 275 6th reference first line: "(1988), Hormonal approaches for studying nervous system development in insects," for "(1988)"
p 275 7th reference first line: delete "In:" once
p 276 2nd reference first line and 3rd line: "Woodburn, T.L. and Tyndale-Biscoe, M." for "Woodburn, T.L., Tyndale-Biscoe, M." and "cyclic" for "Cyclic"
p 276 4th reference 2nd line: "[^{3}H] cholesterol into ecdysteroids by prothoracic glands" for "[^{3}H] Cholesterol into Ecdysteroids by Prothoracic Glands"
p 276 6th reference first line: "Entwicklung" for "entwicklung" and "Engelmann" for "Englemann"
p 277 7th reference first line: "Imagines" for "imagines"
p 277 last reference last line: "*Platysamia*" for : "*Platsamia*"
p 278 3rd reference first line: "Whitten" for "Witten"

ADDENDUM

p xv para 2 line 3: delete "appeared to regulate segmentation..." onwards and read "related to embryonic organogenesis, involving cuticulogenesis and the formation of the gut."

p xv para 3 line 5: delete "appeared to initiate pupariation" and read "were temporally associated with pupariation."

p xv para 4: delete entire paragraph and read "Examination of prepupae revealed that ecdysteroids were associated temporally with the initiation of the larval/pupal moult, while the following decline of ecdysteroid levels was associated with the degeneration of larval tissue. During the pupal stage, ecdysteroids were found to be related to the pupal/adult moult, whereas two distinct very high levels of ecdysteroids during the pharate adult stage coincided with the differentiation of adult tissue and subsequent cuticle secretion, respectively."

p 1 para 1 line 4: delete "To achieve this requirement," and read "Insects have evolved..."

p 2 para 2 end of line 8: read "respectively. Whether these small cell populations deserve the status of a germ layer termed as *entoderm*, however, has been a controversy (Sander *et al.*, 1985)."

p 3 para 2 end of line 1: delete full stop and read "*epidermis*, which lies beneath and secretes the cuticle."

p 3 para 2 line 4: delete entire para from "contains an hydrophobic cement..." onwards and read "consists of three layers: an *inner epicuticle*, an *outer epicuticle* and a *superficial layer*. In many insects, the superficial layer (may be glycoprotein) is covered by a lipid or *wax layer* and, external to this, a variably distinct *cement layer*. The wax layer is vital in preventing dehydration, a function derived from hydrophobic lipid wax. The cement layer, on the other hand, varies in distribution and thickness both between and within species, and is thought to function primarily by protecting the wax layer against abrasive or impact damage. The *outer epicuticle*, a lipoproteinaceous layer, defines the shape and size of an instar, and the *inner epicuticle*, a polyphenol protein-complex layer, is important for integumental wound recovery. The underlying *procuticle* is a composite material consisting of mainly components of a proteinaceous matrix (also including some lipids) and fibre phase of a cellulose derivative, *chitin microfibrils*. The physical and chemical properties of the procuticle are in a constant state of flow which results in the differentiation of procuticle into three components: *exocuticle*, *mesocuticle* and *endocuticle*. The great stability of cuticle generally depends on the degree to which matrix proteins are stabilised by tanning (or sclerotisation) processes (reviewed in Hepburn, 1985).

p 5: delete first sentence last para and read "The degeneration of larval-specific organs has been well analysed cytophysiologically, including the changes in cell plasma and organelles as well as in synthetic capacity of the endocrine glands, during the larval-pupal-adult transformation of *Drosophila melanogaster* (Dai and Gilbert, 1991)."
p16 last para line 2: delete two sentences from "This neuropeptide..." to "(Hua *et al.*, 1997)." and read "Prothoracicostatic activity was demonstrated in larval extracts of the blowfly *C. vicina* by Hua *et al.* (1995) using an *in vitro* ring gland bioassay measuring ecdysteroid biosynthesis. Ecdysiostatic activity was found in two fractions co-migrating with Neb-TMOF (trypsin modulating oostatic factor from *N. bullata*, see section 1.2.2.3.2.). Although the hexapeptide Neb-TMOF was originally isolated from the ovaries

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of adult females and was known as a factor that inhibits egg development, Neb-TMOF also inhibits ecdysteroid biosynthesis in larval moulting glands (Hua *et al.*, 1995). Gel chromatography of brain extracts of *C. vicina* larvae later enabled the isolation of a cerebral factor (11 kDa) with ecdysiostatic activity (Hua *et al.*, 1997). Its possible sequence similarity with Neb-TMOF remains however to be determined. PTSH increases the cAMP level of blowfly ring glands (Hua *et al.*, 1995). These authors suggested that cAMP may control steroidogenesis in blowfly larvae by shutting down ecdysone biosynthesis.

p 31: delete top paragraph and read "At the molecular level, ecdysone initiates moulting via a heterodimeric receptor consisting of EcR (ecdysone receptor) that binds the hormone and USP (ultra spiracle protein) to form a complex that binds to the ecdysone response element on the DNA to activate a number of regulatory genes. The products of these genes both repress ongoing gene expression and stimulate genes associated with the production of the new stage in a cascading fashion. EcR exists in three isoforms: EcRA, EcRB1 and EcRB2. These isoforms contain identical DBD (DNA binding domain) and LBD (ligand binding domain) sequences but differ only in their amino-terminal sequences which are responsible for the differential physiological responses to ecdysone (Riddiford, 1993). The biological roles of these three isoforms have been studied in some details during metamorphosis of *D. melanogaster*. In general, the EcRB1 isoform is expressed mostly in larval cells that are destined to die, while EcRA is expressed predominantly in developing adult tissues (Talbot *et al.*, 1993). However, since EcRB1 is also predominant over EcRA in imaginal histoblast nests and midgut islands and, similarly, EcRA is predominant over EcRB1 in larval prothoracic gland and the above neurons, it is suggested that factors other than EcRA and EcRB1 are also required to specify the metamorphic response to ecdysone. The EcRB2 isoform is a possible candidate. Unfortunately, this cannot be verified until specific antibodies to EcRB2 are available (Talbot *et al.*, 1993).

p 36 para 1: delete entire paragraph from line 5 onwards "Understandably" to "pest management" and read "Understandably, *L. cuprina* has been studied by several research groups mostly on aspects of their ecology, development, reproduction, behaviour and insecticide resistance. The growing awareness of the drawbacks, both financially and environmentally, of conventional pest control programmes e.g. chemical jetting, has renewed interest in the endocrinology of *L. cuprina* which could provide invaluable data for future application in pest management. Although it is unlikely that ecdysteroids are to be used directly in pest management since they are expensive and have little effect when applied topically or orally, knowledge of their biological actions in growth and development can be applied in new approaches to insect control. One approach would be by mimicking or interfering with the action of the hormones using new products that can penetrate the cuticle or enter the body via other ways; another approach would be by blocking binding sites for ecdysteroids on the protein receptors."

p 37 para 2: read below **1.5 Aims** heading and before "This study..." new para "As detailed in section 1.3, the roles of ecdysteroids in insect development have been studied in several dipterans *e.g. N. bullata* (Wentworth and Roberts, 1984), *D. melanogaster* (Märoy *et al.*, 1988) and *P. regina* (Lee, 1992). Although belonging to the same insect order, these three species are from three different families, which differ greatly in their reproduction mode, vitellogenesis, diet, behaviour and development. As expected, this is reflected in their distinct and species-specific ecdysteroid developmental profiles. Although *Lucilia cuprina* belongs to the same family as *P. regina* and may therefore display many similarities with this species, *L. cuprina* is expected to show greater differences with the other two species. Like flesh flies, *L. cuprina* requires a protein meal before vitellogenesis can occur, but differs from the larviparous *N. bullata* by being oviparous. *L. cuprina* also

differs greatly from *D. melanogaster*, not only in terms of behaviour and diet, but also for example in yolk protein biosynthetic pathways. These different developmental and reproductive processes being regulated by complex hormonal interactions, it is therefore important to identify similarities and differences in ecdysteroid profile between those species in relation to the major developmental events."

p 55 end of page: read new heading **2.6 Microscopic techniques** and new paragraph "For a full-body view, the preparations were examined under a Nikon stereomicroscope with a magnification of 0.6 times objectives and 10 times ocular, photographed using Kodak Ektachrome 64 daylight film. For a magnified view, the preparations were examined under a Leitz Orthoplan interference contrast optics microscope with a magnification of 25 times objective and 10 times ocular, photographed using Kodak Ektachrome 160 Tungsten."

p 62: delete entire Fig. 10 caption and read "Fig 10. Dechorionated embryo of *Lucilia cuprina* at 10 - 11 h after oviposition showing the tracheal trunks, the tanning of posterior spiracles (PS) and the appearance of the gut (G). Photographic conditions as for Fig. 7."

p 64 para 2 line 7: delete full stop after "occurred." and read "occurred, and the gut appeared."

p 64 delete entire para 3 and read: "In summary, the single peak of ecdysteroid titre midway during the embryonic development appears concurrently with the initiation of the development of embryonic gut and tracheal trunks, followed by the tanning of mouth hooks and spiracles."

p 76 para 3 last line: read "(Fig 19). Thus the data suggest that photoperiod plays an important role in the PFL stage formation in *L. cuprina*."

p 83 para 4 end of line 3: delete full stop after "spiracles" and read "spiracles, and the appearance of the gut."

p 83 para 4 line 6: delete "initiates" and read "is associated with"

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p 105: delete entire Fig. 33, and read

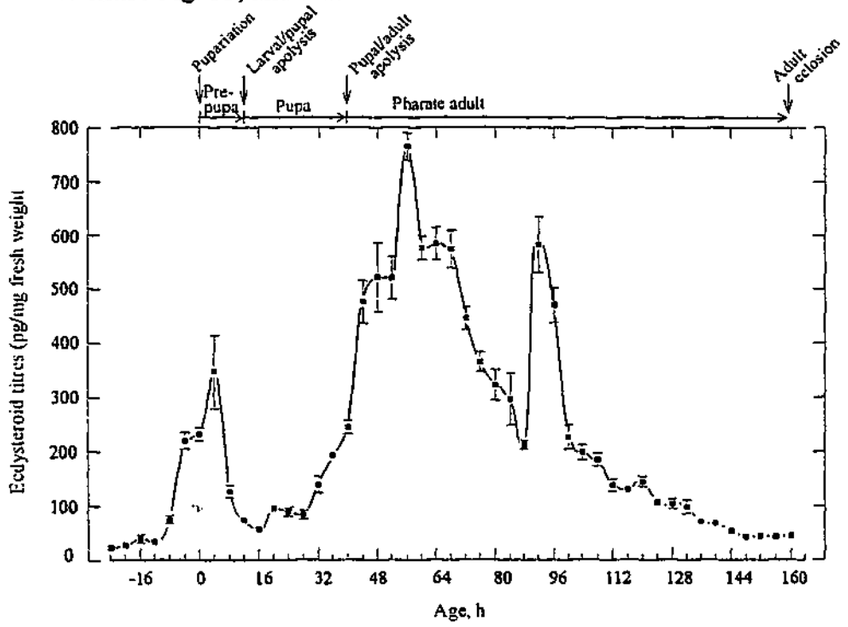

Fig. 33. Whole-body ecdysteroid titres throughout prepupal, pupal and pharate adult stages of development in the females of *Lucilia cuprina* reared at 27° C quantified by RIA. Each point represents the mean titre expressed in pg ecdysteroids/mg fresh body weight ± SEM of 5 individuals, except where the SEM was too small to show.

p 106: delete entire Fig. 34, and read

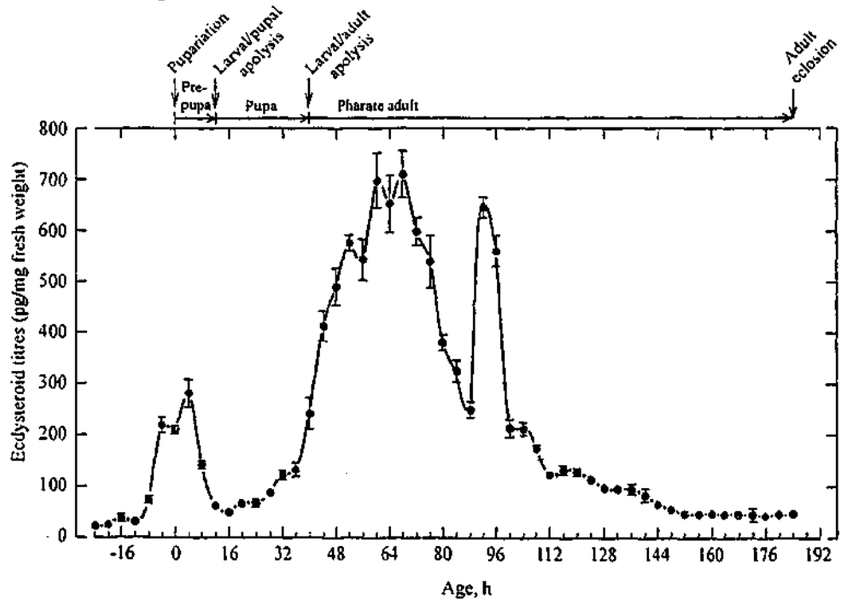

Fig. 34. Whole-body ecdysteroid titres throughout prepupal, pupal and pharate adult stages of development in the males of *Lucilia cuprina* reared at 27° C quantified by RIA. Each point represents the mean titre expressed in pg ecdysteroids/mg fresh body weight ± SEM of 5 individuals, except where the SEM was too small to show.

p 107 delete Table 4 and read:

Table 4. A summary of the time course of development and ecdysteroid levels throughout prepupal, pupal, and pharate-adult of *Lucilia cuprina* reared at 27° C showing coincident developmental events reported in this thesis, as well as data from the literature for *L. cuprina* and related species.

Age (h)	Ecdysteroid levels	Stage of development	Developmental event	Reference for comparison
0		White prepupa	**Pupariation**	
			Increase in granular haemocytes	
			Epidermal cells enlarge	
		Prepupa	Muscles are still striated	
1			Sclerotization of the puparium	
4	Prepupal peak		Disappearence of muscle striations	Barritt and Birt (1971)
8 - 12			**Larval/pupal apolysis**	Fraenkel and Bhaskaran (1972)#
			The stage of cryptocephalic pupa	Carruthers and Roberts (1979)*
			Secretion of the pupal cuticle	
16	Low		Formation of the discs of the imaginal thoracic structures	
			Histolysis of larval muscles	
		Pupa	The pupal cuticle is visible 1 - 2 μ separate from the epidermis of the thorax and its appendages	
			Breakdown of the anterior fat body cells and the basement membrane enclosing the fat body	
24 - 28	Rapid increase			Barritt and Birt (1971)
			Head evagination forms the phanerocephalic pupa	Fraenkel and Bhaskaran (1972)#
			Cuticular expansion, retraction of larval tracheae, migration of brain, tracheae, fat body into the newly formed imaginal structure	
36 - 40	First pharate adult peak (male)			
56				Zdarek and Friedman (1986)#
			Pupal/adult apolysis	
68	First pharate adult peak (female)		Thoracic and leg muscles are growing rapidly	
72		Pharate adult	Formation of flight muscles	
92	Second pharate adult peak		Eye pigmentation	
			Leg differentiation into segments with setae and hair and claws on the feet.	
120				
160	Gradual decrease			
184			Endocuticle deposition	Barritt and Birt (1971)
			Definitive adult morphology	Whitten (1969)#
			Male adult eclosion	Barritt and Birt (1971)
			Female adult eclosion	

* Refers to the flesh fly *Tricholioproctica impatiens*
\# Refers to the flesh fly *Neobellieria bullata*

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p 111 Comment to the examiner: My data show that if we sum up the weight of the carcass and the reproductive system, the result is lower than the average weight of the whole body. This indicates that there was loss of haemolymph. However, a discrepancy occurs when we compare the total ecdysteroids in the whole body with the sum of total ecdysteroids in the reproductive system and the carcass. Some data indicate that the total ecdysteroids in the whole body are lower than in the sum of the reproductive system and the carcass. One possible explanation for this may be high individual variation. This could be reduced by taking a greater number of replicate samples. Another possible explanation is asynchrony, ie variation in developmental stages of the samples. This may be improved by staging the adults based on ovarian developmental stages rather than age. However, as the samples for whole body and for reproductive system and carcass were taken from different individuals, comparison may still remain difficult. It is therefore impossible to say from these data which is the contribution from haemolymph and whether it can be considered negligible or not. Additional experiments measuring the total ecdysteroids in haemolymph and the haemolymph volume would be required.

p 122 para 2: delete line 5 "confined to" and read "in the"

p 127 para 2: delete first sentence and read "The subsequent developmental events (Fig. 10), *ie* the formation of the tracheal trunks and the appearance of the gut, followed by the tanning of the posterior spiracles and mouth hooks, occurred in the same sequence in both *L. cuprina* and *L. sericata* (Davis, 1967)."

p 130 line 7: delete from "As mentioned earlier" onwards and read "Segmentation of the embryo is known to be under the control of many genes. In *D. melanogaster*, at least three nuclear receptors encoded by *kni* (Nauber *et al.*, 1988), *tll* (Pignoni *et al.*, 1990) and *FTZ-F1* genes (Lavorgna *et al.*, 1991) are known to be required for proper initial segmentation of the embryonic body. One of these receptor proteins, FTZ-F1, is implicated in the activation of the segmentation gene, *fushi tarazu* (*ftz*) (Lavorgna *et al.*, 1991). The *FTZ-F1* gene encodes two protein isoforms, $_{\alpha}$FTZ-F1 and $_{\beta}$FTZ-F1 with distinct amino-terminal sequences joined to identical DNA binding domain (DBD) and ligand binding domain (LBD) sequences (Lavorgna *et al.*, 1993). In *D. melanogaster*, $_{\alpha}$FTZ-F1 is expressed only during early embryogenesis. $_{\beta}$FTZ-F1, in contrast, is evenly expressed throughout the blastodermal layer in early embryos as well as in the brain, the ventral cord structures and the hindgut with stronger expression in late embryos (Ohno and Petkovich, 1993). Although the specific ligand of FTZ-F1 is still unknown, the expression of $_{\beta}$FTZ-F1 has been shown to be regulated by ecdysone during larval stages and metamorphosis of *D. melanogaster* (Woodard *et al.*, 1994). However, since this has not as yet been demonstrated in early embryos directly, it is still not clear whether the single peak of ecdysteroids could be responsible for the initiation of embryo segmentation in *L. cuprina*."

p 131: delete entire para 1 and read "Ecdysteroids may also have a significant role in organogenesis. As mentioned earlier, ecdysone expresses its effects on development through its interaction with the ecdysteroid receptor isoforms and USP. Significant levels of an ecdysone receptor LcEcR mRNA were detected in *L. cuprina* embryo (Hannan and Hill, 1997). Unfortunately, the correlation of these receptor isoforms to specific stages of embryogenesis has not as yet been demonstrated in *L. cuprina*. Nevertheless, a complex ecdysone-inducible early gene, *E75A*, which was originally known to be active during *D. melanogaster* metamorphosis, was also found to be required in embryonic gut morphogenesis (Bilder and Scott, 1995). Organogenesis in the present study is indicated by cuticulogenesis, which involves the formation of the tracheal trunks and the tanning of posterior spiracles, as well as the appearance of the gut. These three events occurred 4 to 5 h following the ecdysteroid peak (Table 2), suggesting a possible role of ecdysteroids.

Furthermore, it was demonstrated in *D. melanogaster* that the formation of embryonic cuticle occurred synchronously throughout the organism between 12 and 16 h of embryonic development (Hillman and Lesnik, 1970). Later, Maróy *et al.* (1988) measured the ecdysteroid titre in *D. melanogaster* embryos and found that the ecdysteroid peak occurred at 8 h of embryonic development, i.e. 4 - 8 h before cuticulogenesis. These data are in good agreement with our finding of a free ecdysteroid pulse in *L. cuprina* embryos. The exact role of free ecdysteroid in cuticulogenesis and gut formation, and more generally organogenesis, however still remains to be demonstrated."

p 131: delete entire para 2 (and top p 132 delete para 1) and read "In conclusion, the single peak of free ecdysteroid may perform multiple roles during embryonic organogenesis, through its interaction with its nuclear receptors, presumably expressed differentially at specific stages of development. This mechanism does not, however, exclude the possibility that other hormones reported to be present in embryos such as JH (in *Hyalophora cecropia*, Gilbert and Schneiderman, 1961; in *Oncopeltus fasciatus*, Dorn, 1975, and Bergot, 1981; in *Locusta migratoria*, Roussel and Aubry, 1981) and PTTHs (in *Manduca sexta*, Dorn *et al.*, 1987) may also be present in *Lucilia* embryos. The possible interrelationships between ecdysteroids and other hormones regulating embryogenesis of *L. cuprina* still remain to be investigated."

p 134 end of para 1: read "Thus, the data suggest that a critical weight and photoperiod play important roles in the PFL stage formation in *L. cuprina*."

p 140 last para line 2: delete "tanning and sclerotisation begin and continue" and read "sclerotisation begins and continues"

p 142: delete entire para 2 and read "The temporal association of the prepupal ecdysteroid peak with the initiation of the larval/pupal moult may suggest a possible role of free ecdysteroid in this process, since apolysis started 8 h after pupariation and was completed within the next 4 h (Table 4). Interestingly, the ecdysteroid surge during this stage relates to a different moult *i.e.* larval/pupal (instead of larval/larval). Early in every moult, there is a critical commitment period during which the presence/absence of active JH determines a switch in gene expression in response to the increasing ecdysteroid levels (Riddiford, 1994). This co[illegible]itment period is followed by a pre-differentiative period up to the peak of ecdysteroid le[illegible]"

p 143 line 1: delete "[illegible]steroid level."

p 157 below para 1 read new paragraph "In conclusion, free ecdysteroid may perform multiple roles, through its interaction with its nuclear receptors, presumably expressed differentially at specific stages of development. During *L. cuprina* embryogenesis, a single peak of ecdysteroids may be associated with the initiation of embryonic organogenesis, involving cuticulogenesis and the formation of the gut. During larval stages, ecdysteroid peaks at midway through the duration of the first two larval instars are temporally associated with the first and second larval/larval moults respectively; while in the third instar, extremely high titres of ecdysteroids, at the onset of metamorphosis, are associated with pupariation. As all ecdysteroid peaks during the larval stages occur in the scotophase, the involvement of photoperiod is possible. Exodus of last instar larvae from the food also takes place during this phase. Further experiments would be required to determine whether photophase gating occurs and the exact role of ecdysteroids in this. As previously mentioned, PTTH releases follow a circadian clock regulated by the brain which is entrained by extraretinal photoreception (Vafopoulou and Steel, 1996). It would be interesting to follow the profile of PTTH release in *L. cuprina* to investigate the exact role

of photoperiod in these events. In prepupae, high levels of ecdysteroid are temporally associated with the larval/pupal moult, while the following decline of ecdysteroid levels is associated with larval tissue degeneration. During the pupal stage, ecdysteroids were found to coincide with the pupal/adult moult, whereas two distinct very high levels of ecdysteroids during the pharate adult stage appeared to be associated with adult tissue differentiation and subsequent cuticle secretion, respectively. In adult females, a protein meal is followed by an increase of ecdysteroid levels and the initiation of ovarian development, leading to a significant increase in body weight. In contrast, a protein meal in males is not followed by an increase in ecdysteroid levels, although a slight increase in body weight occurred.

Finally, it is clear that ecdysteroids may perform multiple roles in almost every stage of insect development from the embryogenesis to the adult reproductive phase. As mentioned earlier different EcR isoforms are responsible for the differential physiological responses to ecdysone (Riddiford, 1993). It would be of interest to investigate the expression of LcEcR isoform combinations, as well as other nuclear receptors that are known to be regulated by pulses of ecdysone, at every stage of development to elucidate the more specific roles of ecdysteroids in these various developmental processes."

p 158: delete recipe for Alcoholic Bouin's fixative and read

ALCOHOLIC BOUIN'S FIXATIVE

Alcoholic Bouin's fixative used was made up by mixing of the following compounds:

Picric acid (Crystal)	1 g
Formaldehyde (a 37% solution of formaldehyde gas in water)	60 ml
Acetic acid (glacial)	15 ml
Ethanol (80%)	150 ml

p 158 para 3: delete sentence "All compounds....adjusted to 8.4." and read "All compounds were mixed together and made up to 1 litre with distilled H_20. The pH was adjusted to 8.4 by adding either boric acid if the pH was too high or sodium borate if the pH was too low."

p 243 read after 5th reference:

Bilder, D. and Scott, M.P. (1995), Genomic regions required for morphogenesis of the *Drosophila* embryonic midgut, Genetics 141: 1087 – 1100.

p 262 read before first reference:

Lavorgna, G., Karim, F.D., Thummel, C.S., and Wu, C. (1993), Potential role for a FTZ-F1 steroid receptor superfamily member in the control of *Drosophila* metamorphosis, Proc. Natl. Acad. Sci. USA 90: 3004-3008.

p 278 read after 4th reference:

Woodard, C.T., Baehrecke, E.H., and Thummel, C.S., (1994), A molecular mechanism for the stage specificity of the *Drosophila* prepupal genetic response to ecdysone, Cell: 79: 607-615.

ECDYSTEROID LEVELS AND IMPLICATIONS FOR EMBRYONIC AND POST-EMBRYONIC DEVELOPMENT OF THE BLOWFLY *LUCILIA CUPRINA* (WIED.) (DIPTERA: CALLIPHORIDAE)

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Abbreviations

CA	Corpus Allatum
cAMP	Cyclic Adenosine Monophosphate
CC	Corpus Cardiacum
cDNA	Complementary Deoxyribonucleic Acid
CNS	Central Nervous System
EcR	Ecdysone Receptor
EDNH	Egg Development Neurohormone
EG	Epitracheal Glands
EH	Eclosion Hormone
ESG	Ecdysteroidogenin
ETH	Ecdysis-Triggering Hormone
HPLC	High Performance Liquid Chromatography
JH	Juvenile Hormone
JHB_3	Methyl 6,7,10,11-bisepoxy-3,7,10,11-trimethyl 2,6-dodecadienoate
JHIII	Methyl 10,11-epoxy-3,7,10,11-trimethyl 2,6-dodecadienoate
LNSC	Lateral Neurosecretory Cell
MNSC	Median Neurosecretory Cell
NADA	N-acetyldopamine
NBAD	N-β-alanyldopamine
NSC	Neurosecretory Cell
OMP	Ovary Maturating Parsin
PETH	Pre-Ecdysis-Triggering Hormone
PFL	Post-Feeding Larval Stage
PG	Prothoracic Gland
PTSH	Prothoracicostatic Hormone
PTTH	Prothoracicotropic Hormone
RIA	Radioimmunoassay
TMOF	Trypsin-Modulating Oostatic Factor
USP	Ultraspiracle Protein
WPP	White Prepupal Stage

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Abstract

Whole-body ecdysteroid levels in the Australian sheep blowfly *Lucilia cuprina* were determined using a radioimmunoassay during embryonic, larval, pupal, pharate-adult and adult reproductive stages of development. Cultures were maintained at 27° ± 1° C, 45% RH with a 12D:12L light regime. Developmental events examined at both macroscopic and microscopic levels were correlated with the ecdysteroid titre.

Measurement of ecdysteroid levels during the embryonic stage revealed that a single peak of ecdysteroids approximately one third through the stage appeared to regulate segmentation of the embryo and organogenesis, involving cuticulogenesis and the formation of the gut.

High titres recorded approximately midway through the duration of the first two larval instars appeared to initiate the first and second larval/larval moults respectively; while in the third instar, extremely high titres of ecdysteroids occurred much later throughout the instar, *i.e.* at the onset of metamorphosis, and appeared to initiate pupariation. A possible involvement of photoperiod was detected as all ecdysteroid peaks during larval stages occurred in the scotophase. Exodus of last instar larvae from the food also took place during this phase.

Examination of prepupae revealed that ecdysteroids initiated the larval/pupal moult, while the following decline of ecdysteroid levels resulted in larval tissue degeneration. During the pupal stage, ecdysteroids were found to initiate the pupal/adult moult, whereas two distinct very high levels of ecdysteroids during the pharate adult stage appeared to regulate adult tissue differentiation and subsequent cuticle secretion, respectively.

No significant fluctuation of ecdysteroid level was recorded in both female and male adults fed solely on sugar and water. However, females fed protein-rich material, in addition to this diet, showed a rapid increase in ecdysteroid levels coinciding with the formation of the second ovarian follicle and the start of vitellogenesis, culminating in a prominent peak, just prior to nurse cell degeneration. Thus, the protein meal appeared to result in increase of ecdysteroid levels in adult females and initiated ovarian development, leading to a significant increase in body weight. In contrast, ecdysteroid levels did not increase in males following a protein meal, although a slight body weight increase was recorded.

Statement of Responsibility

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where acknowledged or when due reference is made in the text of the thesis.

Edi Basuki

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Chapter 1

INTRODUCTION

1.1. Insect development

Insect development includes a series of morphogenetic changes which result in the transformation of the developing organisms from an embryo to the adult reproductive state. These exacting physiological processes of growth and maturation demand accurate temporal co-ordination. To achieve this requirement, insects have evolved complex hormonal regulation systems (Truman, 1990), in which the centre of these systems is controlled by both intrinsic and extrinsic stimuli (Bollenbacher and Granger, 1985).

1.1.1. Embryogenesis

Ontogeny of most insects starts inside an egg (Sehnal, 1985) which is enclosed in a protective shell called a *chorion,* produced by the ovarian follicular cells (reviewed in Margaritis, 1985). Penetration of the egg by a sperm initiates meiotic division, resulting in a haploid nucleus that fuses with the sperm nucleus to form a zygote. The next stage is embryogenesis which comprises early embryonic development, blastodermal stage, germ anlage stage, germ band stage, dorsal closure, organogenesis and histogenesis.

Early embryonic development starts with subsequential synchronous mitotic cleavages of the zygote nucleus without complete cell division. These cleavages form a nucleated syncytium on the ventral part of the egg whose outer layer is called the *blastema.* Early embryonic development has been described in some detail for the dipterous *Lucilia sericata* (Fish, 1947a; Davis, 1967) and *Culex fatigans* (Davis, 1967). Cellularisation of this single-layered structure results in the formation of the *blastoderm.* The yolky part enclosed by the

blastoderm is known as the *yolk sac* and consists of the *vitellophages* and the *yolk cells*. The blastoderm also contains cells which are destined to become particular organs in the adult stage. Such groups of cells form rudimental organs in the larval stages called the *imaginal discs* which appear as flat, single or double-layered folds (Oberlander, 1985; Schwalm, 1988). At the end of the blastodermal stage, the blastoderm forms the *germ anlage* (embryonic rudiment) and the *extra-embryonic blastoderm*, which becomes the outer embryonic envelope, the *serosa*.

The germ anlage invaginates along the midline by the process of *gastrulation* to form the two-layered state, the *ectoderm* (outer layer) and the *mesoderm* (inner layer), which leads to the formation of the *germ band* stage. Insects lack an *endodermal layer*. The only comparable structure is the *entoderm* which gives rise to the midgut. The entoderm is equivalent to the vitellophages and yolk cells, or small cell populations initially integrated into the inner layer of the germ anlage, or in the ectodermal invaginations called the *stomodeum* and *proctodeum* which primarily give rise to the foregut and hindgut, respectively (Sander *et al.*, 1985). The germ band stage is externally characterised by segmental organisation (reviewed in Schwalm, 1988). At the end of this stage both lateral and dorsal parts of the germ band expand laterally and meet at the dorsal midline. This process is called *dorsal closure*.

Organogenesis and histogenesis start during the early germ band stages. The mesoderm gives rise to muscles and fat body tissues while sense organs, tracheae, skeletal structures, nervous system, foregut and hindgut, and Malpighian tubules are derived from the ectoderm. The originally uniform embryonic cells in the organs differentiate into tissue-specific cell types during histogenesis, except for the imaginal cells which remain embryonic until late larval or pupal stages. These lengthy processes of histogenesis are completed just prior to hatching (reviewed in Sander *et al.* 1985).

1.1.2. Post-embryonic development

Insect post-embryonic development is characterised by the existence of punctuated stages of development. This arthropod characteristic is caused by the presence of an integument with rigid exoskeleton, the cuticle. To enable growth and development, great structural, physical and chemical changes must occur, especially in the integument from stage to stage.

1.1.2.1. Integument structure and function

The integument consists of a *cuticle* which is secreted by an *epidermis*. After deposition, the cuticle consists of several chemically complex layers, the *epicuticle* and a relatively coarse set of layers composing the *procuticle*. The epicuticle usually contains an hydrophobic cement layer, a predominantly lipid wax layer which is important in waterproofing. The epicuticle includes a lipoproteinaceous *outer epicuticle* that defines the shape and size of an instar, and a polyphenol protein-complex *inner epicuticle* which is important for integumental wound recovery. The procuticle is a composite material consisting of mainly components of a proteinaceous matrix (also including some lipids) and fibre phase of a cellulose derivative, *chitin microfibrils*. The physical and chemical properties of the procuticle are in a constant state of flow which results in the differentiation of procuticle into three components: *exocuticle*, *mesocuticle* and *endocuticle*. The great stability of cuticle generally depends on the degree to which matrix proteins are stabilised by tanning and sclerotisation processes (reviewed in Hepburn, 1985).

The underlying epidermis, which consists of a single layer of typically columnar eukaryotic cells, performs metabolism for self maintenance, synthesis of chemicals and secretion. Epidermal cell activity is regulated by intrinsic and extrinsic factors. Intrinsic factors such as structural asymmetry of individual

cells, thus variations in membrane permeability, age, health, nutritional status and ionic conditions affect activeness and responsiveness (Neville, 1975). Extrinsic factors include humoral and hormonal milieu that permeate through the haemocoel, effects of water, temperature and humidity and also tropic factors and changes related to metamorphosis (Locke, 1990).

1.1.2.2. Moulting

Since the cuticle is a rigid structure, it is a barrier to growth which must be shed as the insect develops, and a new and larger exoskeleton must be produced. This process is called *moulting* (Snodgrass, 1935). Moulting consists of a series of events starting with *apolysis, i.e.* the gradual antero-posterior separation of the epidermis from the cuticle, and ending with *ecdysis, i.e.* the shedding of the old or exuvial cuticle. Apolysis defines the ending of an *instar* and the beginning of the next. The insect within the loosened cuticle is the *pharate* stage until an ecdysis occurs (Jenkin and Hinton, 1966). Following apolysis, the epidermal cells undergo mitotic divisions and folding of the epidermis which lead to a greater template for the newly synthesised cuticle. This is followed by the secretion of the moulting fluids by the epidermal cells (Neville, 1975). The next event is the deposition of a new outer epicuticle at the surface of the apolysed and crenulated epidermis; this layer defines the surface pattern of the new cuticle. A new inner epicuticle is secreted and the moulting fluid enzymes are activated, resulting in lysis and resorption of the old endocuticle. Deposition of the new procuticle then takes place prior to ecdysis (Blum, 1985).

As a moult is completed, a series of developmental events which characterise the *post-moulting stage* begin. During this stage, the new cuticle is expanded and sclerotised. Secretion of endocuticle occurs usually during the photophase. Wax is secreted via pore canals, while deposition of the endocuticle

continues and the apolysial membrane for the next moult forms. The period between the end of post-moulting and the next apolysis is termed *intermoulting*. The complete process of moulting is well documented by Neville (1975) and Hepburn (1985).

1.1.2.3. Metamorphosis

Based on the extent of metamorphosis, insects are generally grouped as *ametabola* for those with no apparent metamorphic changes, *hemimetabola* (with incomplete changes), *paurometabola* (with gradual changes), and *holometabola* (with complete changes). In hemimetabolous insects (*e.g.* cockroaches, grasshoppers) immature stages resemble the adult form, lack a pupal stage and develop their wings and genitalia externally. On the other hand, holometabolous insects (*e.g.* beetles, bees, flies) show larval forms which are morphologically very different from the adult, and an immobile non-feeding pupal stage as an intermediate between the larva and the adult (Blum, 1985).

In the Diptera, after passing through several larval instars, major morphological and physiological changes occur mostly during the last larval and pupal stages. This period of major transformation during which tissues and organs change their structures and functions is called *metamorphosis* (Sehnal, 1985). The imaginal discs, which developmentally arrested during the early larval stages, rapidly differentiate into specific structures (*e.g.* wings, legs) to suit their function for adult life, while the larval-specific organs degenerate (Oberlander, 1985).

Metamorphosis and degeneration of larval specific organs have been well analysed cytophysiologically in the endocrine glands of *Drosophila melanogaster* during the larval-pupal-adult transformation (Dai and Gilbert, 1991). In larvae of cyclorraphous Diptera, including *Lucilia cuprina,* three endocrine glands: the

prothoracic gland (PG), the corpus allatum (CA) and corpus cardiacum (CC), fuse together to form a single glandular composite called the 'ring gland' (Meurant and Sernia, 1993). During metamorphosis, the PG degenerates, and is characterised by: a gradual decrease in its ability to synthesise ecdysteroids; a decreasing quantity of smooth endoplasmic reticulum and mitochondria; the absence of intercellular channels; cytoplasmic fragmentation; and separation of the PG from the CA and CC. Just before and after adult eclosion, the PG becomes remnant, while the CA migrates to its position as a separate and active gland, which is indicated by high concentration of smooth endoplasmic reticulum, mitochondria, and mitochodrion-scalariform junction complexes (Dai and Gilbert, 1991).

A unique feature of metamorphosis in most Diptera, including *L. cuprina,* is that the transformation from larval to adult stage is interposed by a pupal stage which lies inside a barrel-like structure, the *puparium*, which is formed from the cuticle of the third (*i.e.* the last) larval instar. The early stage of this structure is called the *white puparium* until its cuticle tans (Fraenkel and Bhaskaran, 1973). The process which leads to the formation of the puparium is called *pupariation.* During this process, the larva irreversibly ceases feeding and the *post-feeding larva* gradually retracts longitudinally, resulting in a barrel shape with smooth surface called the *white prepupa* (Zdarek and Fraenkel, 1972). The stage between the completion of the white puparium formation and the following larval/pupal apolysis is the *prepupal* stage (Metcalf and Flint, 1932). During this period the animal is no longer a larva but not yet a true pupa. Following larval/pupal apolysis and the secretion of pupal cuticle, the animal is in the stage of a *cryptocephalic pupa,* until the head is evaginated by a process of muscular contractions in the abdomen producing the *phanerocephalic pupa* (Zdarek and Fraenkel, 1972). Pupal/adult apolysis takes place a relatively long time after the

formation of the phanerocephalic pupa, and results in the formation of the *pharate adult* until emergence, when the adult fly sheds both larval and pupal cuticles simultaneously (Jenkin and Hinton, 1966).

1.1.2.4. Development of gonads and sexual maturation

After adult emergence and cuticle sclerotisation, the dipteran imago is able to feed and fly. It takes some time, however, before sexual maturation is completed especially in females (Sehnal, 1985). Early adult life is still developmentally active, and is characterised by the maturation of gonads and the morphogenesis of other tissues. This includes the degeneration of remnants of larval organs such as abdominal intersegmental muscles and fat body, and the continuous formation and development of imaginal organs like the flight muscles and new fat body (Chapman, 1969).

1.1.2.4.1. Females

The internal female reproductive system of *L. cuprina* consists of a pair of ovaries, paired lateral oviducts converging into a common oviduct, three spermathecae and a pair of tubular accessory organs. The paired ovaries consist of ovarioles, each of which is made up of a series of developing follicles. Follicles are formed when individual oöcytes are bordered by mesodermal cells that differentiate into follicle cells. The distal part of each ovariole differentiates into a germarium which contains primary oogonia. The proximal part of ovariole differentiates into vitellarium where follicles, each containing one oöcyte, move toward the oviduct. The region which contains the follicles in each ovary is connected to the common oviduct by the lateral oviduct. The lateral oviduct is closed from the first follicle by an epithelial plug until the mature egg is released from the follicle. The most mature follicle suppresses the younger follicles from further growth until the oldest egg is fully mature and laid (Vogt *et al.*, 1974).

Ovarian developmental stages in the blowfly *L. cuprina* has been described by Clift (1972) using a 10-stage scale adapted from Adams and Mulla (1967) for *Musca domestica*. The cycle of ovarian development begins with stage 1 where only the germarium is visible under light microscopy. In stage 2 a secondary follicle with a spherical shape is produced and in stage 3 the developing follicle becomes translucent and ovate with the oöcytes clearly distinguishable. Stage 4 is characterised by the onset of vitellogenesis and cuboidal follicle cells over the nurse cells. The amount of yolk progressively increases and during stage 5 it occupies approximately one-third of the follicle, while the follicle cells over the nurse cells become squamous. In stage 6 the vitelline membrane starts to form and the yolk occupies up to half of the follicle. In stage 7 the yolk occupies between half and three-quarters of the follicle, while in stage 8, the yolk occupies more than three-quarters of the follicle and the degeneration of the nurse cells starts. The completion of yolk deposition and the onset of chorion formation occur during stage 9. The egg is fully mature at stage 10 as indicated by the completion of chorion deposition and the formation of the hatching pleat. For convenience of age determination of *L. cuprina* females, Vogt *et al.* (1974) used a 6-stage scale (O to V). The correspondence between the stage criteria used by Clift (1972) and Vogt *et al.* (1974) is as follows: Stages 1=O, (2 and 3)=I, (4 and 5)=II, (6 and 7)=III, (8 and 9)=IV, 10=V.

Ovarian development and sexual maturation can be completed only when environmental conditions (*i.e.* diet, photoperiod, temperature, *etc.*), as well as hormonal conditions, are favourable. Wild-type (*i.e.* anautogenous) *L. cuprina* females require a sufficient quantity of protein-rich diet to allow yolk deposition to proceed during ovarian development (Barton-Browne *et al.*, 1979). On the

other hand, some females are able to mature at least some oöcytes without protein ingestion *i.e.* autogenous (Barton-Browne *et al.*, 1981).

Ingestion of protein-rich material increases sexual receptivity in both autogenous and anautogenous female *L. cuprina* (Barton-Browne *et al.*, 1976). Protein-fed females are, however, able to mature eggs without mating (Barton-Browne *et al.*, 1980). In autogenous females, mating appears to increase the number of mature eggs as the protein of the male accessory gland fluid may provide an additional nutritional supply (Barton-Browne *et al.*, 1981). In contrast, anautogenous females do not receive any obvious material benefits at mating beyond the sperm (Cook, 1995).

Protein deficiency in anautogenous *L. cuprina* females results in the cessation of ovarian development either in stage 5(II) or earlier. If sufficient or excessive protein is ingested, on the other hand, they are able to mature eggs (Barton-Browne *et al.*, 1979). A similar phenomenon was found by Clift and McDonald (1976), *i.e.* if a female cannot develop a complete batch of eggs, its ovarian development will cease usually at the beginning of yolk deposition. This discontinuity of the relationship between the quantity of protein-rich material ingested and the stage of ovarian development led these authors to hypothesise that for ovarian development to proceed to stage 6 (III) or beyond, specific hormonal conditions are necessary. The release of these hormonal requirements is triggered by the ingestion of an appropriate amount of protein-rich material (Barton-Browne *et al.*, 1979).

When adult females (both autogenous and anautogenous) do not acquire sufficient protein-rich material, they mature only some of their oöcytes and the rest are resorbed in early vitellogenesis. Oöcytes resorption, however, does not appear to be a direct response to the lack of nutrient, but rather a response to a

warning of protein-deficiency condition (Barton-Browne *et al.*, 1979). These authors suggested that signals such as concentration of vitellogenin and/or of one or more hormones in the haemolymph, may regulate the degree and timing of oosorption.

1.1.2.4.2. Males

In *L. cuprina,* the internal male reproductive system consists of a pair of testes, a pair of vasa deferentia, a pair of tubular accessory glands and a single seminal duct with a pumping organ. Each testis consists of a single orange pigmented follicle and the two exiting vasa deferentia fuse to form the ejaculatory duct. The pumping organ which is located at the posterior end of the seminal duct consists of a sclerotised disc, the ejaculatory apodeme, and an unsclerotised diverticulum with a valve system (Clift and McDonald, 1973).

Newly emerged males have mature sperm, and their maturation is related mainly to the functional activation of the accessory glands (Sehnal, 1985). At this stage, testes which are ovoid in shape and orange in colour, contain sperm bundles which separate 6 - 8 h later. Non-motile sperm bundles are located in the distal bulb of the testis whereas motile sperm cells are located in the basal bulb. On the second day the testes change into a pyriform shape and become darker in colour.

In some insect orders, males possess a spermatophore to transfer sperm during copulation. This sperm supply is available to the female for later insemination. In Diptera, however, the transfer of sperm is direct (without a spermatophore), and insemination is aided by male secretions and ejaculation contractions of the accessory glands resulting in the deposition of sperm into the spermathecae of the female (Smith *et al.*, 1988).

1.2. Endocrinological aspects of insect development

1.2.1. The classical scheme of insect endocrine system

Studies on hormonal control of insect development can be traced back from the nineteenth century. The pioneering work by Kopec (1917) showed through surgical and ligation experiments that the brain of the gypsy moth *Lymantria dispar* secreted a humoral factor required for metamorphosis. This finding was corroborated by Wigglesworth (1934, 1936) who demonstrated that when fourth instar larvae of the blood-sucking bug *Rhodnius prolixus* were decapitated, some larvae failed to follow the usual developmental pattern, some moulted into fifth instar larvae while only a few of them developed adult structures. This clearly showed that the process was regulated by a chemical factor. This finding was also confirmed in Diptera by Fraenkel (1935) who demonstrated that last instar larvae of *Calliphora erythrocephala* ligated in the middle, pupariated only in the anterior sections. The posterior parts could be induced to pupariate by injecting haemolymph from pupariating larvae. It was suggested by Hanstrom (1938) that the neurosecretory cells (NSCs) in the dorsal part of the brain could be the source of the hormone in *Rhodnius*, and this was corroborated by Wigglesworth (1940) when decapitated larvae of *Rhodnius* were induced to moult by implantation of this part of the brain.

Through experiments during 1939 and 1940, Wigglesworth found that adult *Rhodnius* could produce a new cuticle under an exposure to the moulting hormone by parabiosis with fifth-instar larvae. But if the moulting adult was implanted with CA, the new cuticle showed a recovery of larval characters. This was called reversal of metamorphosis. Wigglesworth then proposed a name for the hormone produced by the CA which had been known as the "inhibiting hormone", the *juvenile hormone* (JH).

It was not until 1940 that the importance of the PG in the process of moulting was demonstrated by Fukuda, using a series of organ transplant experiments in *Bombyx mori*. Fukuda showed that ligated abdomen could be induced to pupate by implantation of PG. He concluded that the PG were the source of a moulting hormone.

In 1942, Piepho proposed that cerebral neurosecretion activates the PG to secrete the moulting hormone in *Galleria mellonella*; and this idea was proved experimentally by Williams (1947) in *Hyalophora cecropia* and confirmed in *R. prolixus* by Wigglesworth (1952). These findings of the interaction between the brain, the CA and the PG and their products form the "classical" scheme for insect endocrinological control.

1.2.2. Major hormones involved in the control of insect development (other than ecdysteroids)

It is now known that the insect brain, in response to environmental stimuli, integrates growth, development, and reproduction regulation, which involves numerous sensory receptors and nerve transmission, in part by synthesising and releasing cerebral *neurohormones* via neurohaemal organs (CC or CA), controls the synthesis of the two groups of insect developmental hormones, the *ecdysteroids* and the *JHs*. Furthermore, non-cerebral factors are known to be involved in insect development *i.e. ecdysis-triggering hormone* (ETH) and ovarian development *i.e. folliculostatins* (reviewed in Gäde *et al.*, 1997). Their biochemistry and function will be discussed briefly in the following sections.

1.2.2.1. Neurohormones

Neurohormones are peptides produced by NSCs that regulate physiological, developmental, and behavioural events in insects. Major members of these peptides, *prothoracicotropic hormones* (PTTHs), *prothoracicostatic hormones* (PTSHs), *eclosion hormones* (EHs), and *bursicon* are primarily involved in the control of moulting and metamorphosis, while others such as *folliculostimulins* and *allatotropins* are involved in the control of ovarian development (reviewed in Gäde *et al.*, 1997).

1.2.2.1.1. Prothoracicotropic hormones (PTTHs)

PTTHs stimulate the PG to synthesise and release ecdysone, and are therefore a key hormone for the regulation of insect moulting and metamorphosis (Watson *et al.*, 1989). PTTHs are secreted by NSCs, specialised unipolar neurones in the insect brain (Hokfelt *et al.*, 1980). Based on location, size, number of cells and sites of axon termination, so far four groups of NSC have been identified in the brain of *Manduca*. Using a bioassay, it was shown that the group III lateral NSC (L-NSC III) have prothoracicotropic activity (Agui *et al.*, 1979). This was then confirmed using immunocytological staining by O'Brien *et al.* (1988). In *Manduca*, the paired somata of NSCs are located dorso-laterally in each protocerebral hemisphere. Their axons cross, extend and exit the brain, traverse the CC and the CA where they branch expansively over the organ to form a neurohaemal structure for PTTH release (Westbrook and Bollenbacher, 1990).

PTTHs appear to be a group of structurally related neuropeptides that can be classified in two main forms, according to their molecular sizes, as either big or small. These neuropeptides have been isolated from several species. The big *Bombyx* PTTH is a 30 kD homodimeric glycoprotein (Ishizaki and Suzuki, 1994). The small (4.4 kD) form of *Bombyx* neuropeptide (Nagasawa *et al.*, 1986) does

not appear to have prothoracicotropic activity in *Bombyx* itself, rather, it does in a different species (*e.g. Samia cynthia ricini,* Ishizaki *et al.,* 1983). For this reason, small *Bombyx* PTTH is called bombyxin (Mizoguchi *et al.,* 1987). A small (~7 kDa) and big PTTHs (~25-30 kDa) have also been isolated from *Manduca sexta* (Muehleisen *et al.,* 1994), while a small PTTH (~2.1 kDa) was isolated from *Lymantria dispar* (Kelly *at al.*, 1995). Bombyxin is so far the best characterised among insect neuropeptides. Its chemical structure is a dimer of identical peptide subunits linked by disulphide bonds, which have a structural similarity to vertebrate insulins, especially in the number and position of cystein residues (Nagasawa *et al.,* 1984; Nagasawa *et al.,* 1986). Bombyxin is synthesised by eight dorsomedial NSCs of brain (Ishizaki and Suzuki, 1994).

In Diptera, a single (~45 kDa) PTTH was isolated and characterised from extracts of whole larvae of *D. melanogaster* using HPLC. Biological activity was tested using a ring gland *in vitro* assay in which ecdysteroidogenesis was measured by radioimmunoassay (RIA) (Kim *et al.*, 1997). In another dipteran species, on the other hand, two fractions (a big ~16 kDa and a small ~5 kDa), that could stimulate ecdysone secretion by ring glands *in vitro,* were separated by gel chromatography from brains of blowfly larvae *C. vicina* (Hua *et al.,* 1997).

Regulation of PTTH release

In general, PTTH release is regulated by both extrinsic and intrinsic stimuli transmitted by either neural and/or endocrine mediators. These stimuli can be classified into three categories: (1) proprioceptive or mechanoreceptive, (2) photoperiodic, both circadian and seasonal and (3) seasonal changes in temperature (reviewed in Bollenbacher and Granger, 1985). These stimuli are integrated by the brain which responds, thereby regulating the timing of PTTH

release. The response is presumably transmitted to the NSCs which produce PTTH via synaptic connections (Steel and Davey, 1985).

Proprioceptive stimuli play an important role in controlling PTTH release which is expressed in the initiation of moulting. In the haematophagous bug *R. prolixus* (Wigglesworth, 1934) it was found that not only sufficient nutrition but also adequate distension of the abdominal wall was needed to initiate moulting. In cyclorraphous Diptera stimulatory inputs from the crop are prerequisites for pupariation in *Sarcophaga argyrostoma* (Zdarek and Fraenkel, 1970), while in *S. peregrina* wet conditions inhibit pupariation (Ohtaki *et al.*, 1968) and ecdysteroid secretion (Ohtaki *et al.*, 1968; Zdarek and Fraenkel, 1970). Zdarek and Fraenkel (1971) suggested that PTTH release is delayed because the crop of wet larvae is not able to contract while it retains a gas bubble until transferred to a dry environment.

Photoperiodic regulation of PTTH release in late larval, prepupal and pupal stages of *Sarcophaga bullata* (now renamed *Neobellieria bullata*) was demonstrated by Roberts (1984). This author suggested that the occurrence of post-feeding larvae is controlled by photoperiod whose stimulus is passed on to the brain, which results in the release of PTTH and stimulation of the PGs to secrete ecdysteroids. This situation also occurs in *D. melanogaster* (Roberts *et al.*, 1987). A daily rhythm of release of PTTH has been reported in *Rhodnius prolixus* (Vafopoulou and Steel, 1996). Using an *in vitro* technique, PTTH released by explanted brain-retrocerebral was bioassayed using its ability to stimulate ecdysteroid synthesis in arrhythmic PGs. The rhythm free-runs in both continuous darkness (DD) and continuous light (LL) with a period length close to 24 hr. The rhythm appears to damp out more rapidly in LL than in DD. This

indiates that the circadian clock regulating PTTH release is in the brain and is entrained by extraretinal photoreception.

The role of temperature stimuli in the control of PTTH release is exemplified in *D. virilis* by Rauschenbach *et al.* (1987). They suggested that extreme adverse conditions (high or low temperatures) cause delays in metamorphosis until the unfavourable conditions pass. This suggests that extreme temperatures cause inhibition in the ability of the NSCs to secrete PTTH.

Furthermore, it would appear that the termination of PTTH release is controlled by a feed-back mechanism from the endocrine changes initiated by PTTH. The hormonal effects of ecdysteroids (Marks, 1972; Steel, 1975; Agui and Hiruma, 1977) as well as JH (Fukaya and Kobayashi, 1966; Yagi and Fukaya, 1974) on the cerebral NSCs regulate the release and/or synthesis of PTTH. Ecdysteroids and JH themselves, however, are controlled by mechanisms which dynamically integrate intrinsic and/or extrinsic cues. Therefore, the basis of the regulation of development is the dynamic interaction of the insect with its ever changing environment to insure its survival (Koolman, 1995).

1.2.2.1.2. Prothoracicostatic hormone (PTSH)

PTSH is a cerebral factor which inhibits ecdysteroid biosynthesis in the PGs. This neuropeptide was first isolated from the blowfly *C. vicina* and identified as an hexapeptide (Hua *et al.*, 1995). Through an investigation by gel chromatography of brain extract, a fraction (11 kDa) with ecdysiostatic activity was detected (Hua *et al.*, 1997). PTSH increases the cAMP level of blowfly ring glands. These authors suggested that cAMP may control steroidogenesis in blowfly larvae by shutting down ecdysone biosynthesis.

1.2.2.1.3. Eclosion hormone (EH)

EH is a neuropeptide hormone produced by ventro-medial NSCs in the brain (Truman, 1990), which functions in co-ordinating the events of ecdysis (Truman and Riddiford, 1970). Precise temporal co-ordination of ecdysis is crucial for a successful moulting process (Truman, 1990). It was not until 1970 that the first evidence indicating that events during ecdysis were under hormonal co-ordination was shown using giant silkmoths (Truman and Riddiford, 1970). Extirpation and reimplantation of the brain proved that the brain could control the timing of ecdysis. A humoral link mediated the communication between the brain and the rest of the central nervous system (CNS) when the brain was transplanted into the abdomen. Investigations of this neurohormone (Truman, 1971) have focused mainly on lepidopterans: *e.g.* giant silkmoths (Truman, 1971, 1976, 1978), and the tobacco hornworm, *M. sexta* (Reynolds *et al.*, 1979). Various bioassays based on the property of EHs in initiating ecdysis have been used to isolate and eventually sequence the EHs from *B. mori* (Kono *et al.*, 1987) and from *M. sexta* (Kataoka *et al.*, 1987b). The results indicate that EH is a peptide hormone comprised of a single amino acid chain of 62 residues and is encoded by a single gene (Horodyski *et al.*, 1989).

1.2.2.1.4. Bursicon

Bursicon is a neurohormone known to have a role in the process of tanning of the new cuticle. This hormone was first found in the adult blowfly *C. erythrocephala* by Cotrell (1962) and Fraenkel and Hsiao (1962). Using a bioassay, bursicon was found to be massively released into the haemolymph during the period of rapid post-ecdysial tanning of the adult. Bursicon also appears to control tanning and melanisation in larval stages of *Schistocerca gregaria* as demonstrated by Padgham (1976). However, bursicon could not be

detected in the larval stage of *N. bullata* (Roberts *et al.*, 1982). More recently a protein of ~30 kDa with bursicon activity from *Tenebrio molitor* was eluted by gel electrophoresis at pH 5.65, consisting of a single chain (Kaltenhauser *et al.*, 1995). The same technique was applied to homogenates of either the ventral or abdominal nervous system of *C. erythrocephala, P. americana, G. bimaculatus* and *L. migratoria.* In all these species, bursicon activity assayed in ligated blowflies *C. erythrocephala,* was associated with a protein with a molecular mass of 30 kDa (Kostron *et al.*, 1995).

1.2.2.2. Ecdysis-triggering hormone (ETH)

ETH, a peptide hormone that acts in concert with EH on ecdysis was first reported by Zitnan *et al.* (1996). This hormone is produced by epitracheal glands (EG) in larvae, pupae and adults of *M. sexta* that contain mostly cellular component, so called Inka cells. Mas-ETH which is described from the tobacco hornworm *M. sexta*, contains 26 amino acids. EG extract or synthetic Mas-ETH initiates pre-ecdysis within 2 to 10 minutes, followed by ecdysis when injected into larvae, pupae, or adults. Sensitivity to Mas-ETH appears much earlier, before ecdysis, and occurs with shorter latency than that reported for EH. The isolated central nervous system responds to Mas-ETH, but not to EH, with patterned motor bursting corresponding to *in vivo* pre-ecdysis and ecdysis. These phenomena led the authors to suggest that Mas-ETH may be an immediate blood-borne trigger for ecdysis through a direct action on the nervous system.

Furthermore, the gene encoding for a pre-ecdysis-triggering hormone (PETH) and ETH have been identified in Inka cells (Zitnan *et al.*, 1999). Prior to the behavioural onset of ecdysis, rising ecdysteroid levels induce expression of the ecdysone receptor (EcR) and ETH genes in Inka cells and stimulate the CNS

sensitivity to PETH and ETH. Subsequent ecdysteroid decline is required for peptide release, which initiates three motor patterns in specific order: PETH triggers preecdysis I, while ETH activates preecdysis II and ecdysis.

In Diptera, an *eth* gene encoding peptides, with ETH-like structure and biological activity in *D. melanogaster* has been identified producing three putative peptides based on canonical endopeptidase cleavage and amidation sites (Park *et al.*, 1999). Two of the peptides (DrmETH1 and DrmETH2) induce premature eclosion upon injection into pharate adults.

1.2.2.3. Hormones involved in ovarian development regulation

1.2.2.3.1. Folliculostimulins

Folliculostimulins are cerebral factors that stimulate ovarian development. These factors seem to be species specific, *e.g. egg development neurohorme* (EDNH) in *A. aegypti* (Lea, 1972), ecdysteroidogenin (ESG) in *M. domestica* (Adams *et al.*, 1997), and *Locusta migratoria ovary maturating parsin* (Lom-OMP) in *Locusta migratoria* (Richard and Girardie, 1992). In the latter species, the single polypeptide of 65 amino acids is synthesised by the P-median NSCs (Richard and Girardie, 1992). This peptide triggers its gonadotropic effect by inducing the ovary to produce ecdysone, thus acting as a putative ecdysiotropin.

In higher Diptera, a follicle stimulating factor, ESG, was purified from head extracts of the housefly *M. domestica* (Adams *et al.*, 1997). Using HPLC revealed ecdysteroidogenic activity from a fraction with a molecular mass of 8.1 kDa. ESG injected into flies without the CA-CC complex stimulated ovarian development to late vitellogenesis. ESG was also found to stimulate maximal ovarian ecdysteroid production *in vitro*. This is clear evidence that ESG acts as an ecdysiotropin.

1.2.2.3.2. Folliculostatins

Folliculostatins *i.e.* factors that inhibit egg development, such as trypsin-modulating oostatic factor (TMOF), have been demonstrated in several insect species, including flies. Several mechanisms of inhibition have been suggested (reviewed by Gäde *et al*, 1997).

In Diptera, a decapeptide hormone with a molecular weight of 1,047.6 that distinctly inhibits the biosynthesis of trypsin- and chymotrypsin-like enzymes in epithelial cells of the midgut and indirectly inhibits vitellogenesis in anautogenous female mosquitoes *A. aegypti* has been found by Borovsky and colleagues (1993). Aea-TMOF is synthesised and secreted by follicular epithelium cells of the ovary.

In higher Diptera, at least two folliculostatins have been reported from the flesh fly *Neobellieria bullata*: one named Neb-TMOF, a hexapeptide which has a 75 kDa protein precursor (Bylemans *et al.,* 1995) and more recently, *collostatin*, a folliculostatin which has the amino acid sequence of H-SIVPLGLPVPIGPIVVGPR-OH (Bylemans *et al.,* 1998) and so named because of its sequence similarities with parts of several known collagens and its oostatic activity.

1.2.2.4. Juvenile hormones (JHs)

JHs appear to have various functions throughout the life cycle of insects including development, reproduction and behaviour. JH is now known to be a series of homologues produced by the CA. These have been characterised from various species: JHI from *H. cecropia* by Roller *et al.* (1967), JHII from the same species by Meyer *et al.* (1968), JHIII from *M. sexta* by Judy *et al.* (1973), JH0 and 4-methyl JHI by Bergot *et al.* (1980) from eggs of *M. sexta,* and lastly JHB_3 bisepoxide in larvae of *D. melanogaster* by Richard *et al.* (1989) and in larvae and

adults of *L. cuprina* by Lefevere *et al.* (1993). All these hormones have a common sesquiterpenoid skeleton. Extraction and purification from Lepidoptera led to the identification of JHI as methyl 10,11-epoxy-7-ethyl-3,11-dimethyl-trans-2,6-tridecadieonate and JHII as a 17-carbon homologue and JHIII is a 16-carbon homologue (reviewed in Schooley and Baker, 1985). Although some JH homologues seem to be stage-specific, most species from one order appear to possess the same JH homologue. In Diptera, however, mosquitoes have the JHIII homologue (Baker *et al.*, 1983), whereas flies appear to have JHB_3 as the main homologue (Richard *et al.*, 1989; Lefevere *et al.*, 1993).

1.2.2.4.1. Allatotropins

Neuropeptides that either stimulate (allatotropin) or inhibit (allatostatin) JH production have been isolated from the brains of several insect species. The first study on allatotropic factors was carried out with larvae of *M. sexta*: brain extracts seemed to contain factors that stimulated JH synthesis (Granger *et al.*, 1984). In 1989, Kataoka *et al.* isolated and identified a tridecapeptide from heads of pharate adult *M. sexta* that activated JH synthesis. Allatotropic factors have now been characterised in several species. For example, the brain of last larval instar *G. mellonella* contains a 20 kDa polypeptide that stimulates JH synthesis *in vitro* (Bogus *et al.*, 1994), whereas 0.7 kDa – 2 kDa allatotropins were isolated from female and male brain-CC complex of *Locusta migratoria* (Lehmberg *et al.*, 1992).

In Diptera, Toyoda *et al.* (1999) removed the MNSC (median NSC) of the brain-retrocerebral complex of female adults of the blowfly *Protophormia terraenovae* to investigate the role of the brain in ovarian development and diapause. These authors suggested that the MNSC may secrete an allatotropic factor that stimulates vitellogenesis. Severance of the nervi corporis cardiaci,

cardiac recurrent nerve and removal of the CC and hypocerebral ganglion complex, indicated that an allatotropic factor seems to be released into the haemolymph from the hypocerebral ganglion complex and a part of the aorta.

1.2.2.4.2. Allatostatins

Neuropeptides with allatostatic activity have been isolated and characterised from brain extracts of various cockroach species (*Diploptera punctata, Periplaneta americana* and *Blatella germanica*) and in cricket (*Gryllus bimaculatus*), representing a family of allatostatins (Tyr/Phe-Xaa-Phe-Gly-Leu/Ile-NH2) (Gäde *et al.*, 1997). In *G. bimaculatus,* four neuropeptides with sequence similarity to cockroach allatostatin at the COOH terminus (Gly-Xaa-Trp-NH2) and a common amino acid at position 2 (Trp) have been isolated. These peptides have been designated as *G. bimaculatus* allatostatic neuropeptides B1-4 (Grb-AST B1-4) on the basis of their ability to inhibit juvenile hormone III biosynthesis by cricket CA (Lorenz *et al.*, 1995)

In Diptera, allatostatins have been studied in several species. Eight neuropeptides with COOH-terminal amino acid sequence homology to the cockroach allatostatins, have been identified in the blowfly *C. vomitoria.* Four of these peptides end COOH-terminally in Tyr-Xaa-Phe-Gly-Leu-NH_2 and were named Leu-callatostatins. The fifth peptide has a COOH-terminal Met instead of Leu and was named Met-callatostatin (Duve *et al.*, 1993). Bioassays have revealed that these callatostatins from the blowfly have potent allatostatic activity in the cockroaches, *D. punctata, P. americana,* and *B. germanica.* However, these peptides do not inhibit synthesis of JHIII bisepoxide in the fly itself. Another three post-translationally modified Met-callatostatins have been identified from head extracts of *C. vomitoria.* [Hyp^3]Met-callatostatin and [Hyp^2]Met-callatostatin are hydroxylated analogues of Met-callatostatin; the third

one represents a truncated hexapeptide, des Gly-Pro Met callatostatin (Duve *et al.*, 1995). The prohormone gene encoding the Leu-callatostatin peptides has been isolated from *C. vomitoria* genomic DNA. The library and its homologue was cloned from genomic and cDNA libraries of *L. cuprina* (East *et al.*, 1996). Both gene and prohormone structure and organisation are essentially identical in the two species. The prohormone is 180 amino acids long and contains 2 blocks of tandemly arranged Leu-callatostatin peptides with 5 copies of the COOH-terminal sequence –YXFGL. Using information from the Leu-callatostatin gene sequences of the blowflies *C. vomitoria* and *L. cuprina,* antisera specific for the variable post-tyrosyl amino-acid residues Ser, Ala and Asn of the common Leu-callatostatin C-terminal pentapeptide sequence -YXFGL-NH2 were developed (Duve *et al.*, 1996). RIAs based on these antisera were used to purify peptides from blowfly heads. In this way, five neuropeptides of the Leu-callatostatin family were identified. Three have a seryl residue in the post-tyrosyl position. Two of these are octapeptides that differ only at the N-terminal residue; NRPYSFGL-NH2 and ARPYSFGL-NH2 whilst the third is the heptapeptide derived by N-terminal trimming; RPYSFGL-NH2. The other two are also octapeptides in which X is Ala and Asn: VERYAFGL-NH2 and LPVYNFGL-NH2. Experiments on the inhibitory effects of these peptides on the spontaneous contractile activity of the blowfly rectum suggested that regulation of gut motility in insects, rather than allatostatic function, may represent ancestral and universal function of the allatostatins.

1.2.2.5. Ecdysteroids

1.2.2.5.1. Chemistry

Butenandt and Karlson (1954) first succeeded in obtaining 25 mg of pure crystalline ecdysone from half a ton of *B. mori* pupae. A larger amount of 250 mg of the pure hormone was obtained by Karlson (1960) from one ton of *B. mori* pupae. With the increasing availability of the material, these authors identified the chemical structure of ecdysone as a steroid containing a conjugated ketone grouping and five hydroxy groups (Karlson *et al.*, 1963). This structure was proven using X-ray stereochemistry analysis (Hüber and Hoppe, 1965) and the compound was formally termed as 2β,3β,14α,22R,22-pentahydroxy-5β-cholest-7-en-6-one or α-ecdysone (Fig. 1 A). In tissues such as body wall, fat body, gut, Malpighian tubule, ecdysone is converted into the physiologically active form, 20-hydroxyecdysone (Fig. 1 B) or β-ecdysone by the enzyme system 20-monoxygenase (Smith and Mitchell, 1986).

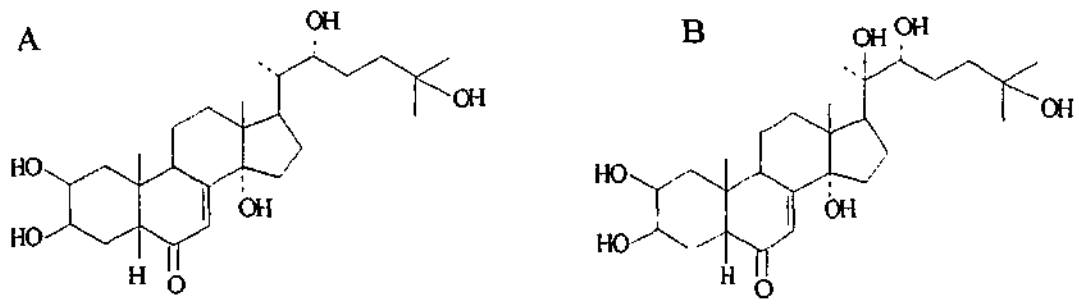


Fig. 1. Chemical structure of ecdysone (A) and 20-hydroxyecdysone (B). Redrawn after Feldlaufer *et al.* (1984).

20-Hydroxyecdysone has also been isolated from various arthropods (Horn and Bergamasco, 1985) and it is considered to be formed from ecdysone (Horn *et al.*, 1966). Numerous derivatives of ecdysone (α-ecdysone) and 20-hydroxyecdysone (β-ecdysone) have subsequently been isolated from both animals and plants (Horn and Bergamasco, 1985) and they are collectively called "ecdysteroids" (Goodwin *et al.*, 1978).

1.2.2.5.2. Sources

During the larval stages, the PGs are the source of ecdysteroids. Evidence that the PG releases ecdysteroids has been directly demonstrated, using *in vitro* culture of this organ in suitable media, from several insect species: *M. sexta*, (King *et al.*, 1974), *Leucophaea maderae* (Borst and Engelmann, 1974; King and Marks, 1975), *S. bullata* (Bollenbacher *et al.*, 1976; Roberts *et al.*, 1984), and *L. migratoria* (Hirn *et al.*, 1979).

It appears that several other tissues can be alternative sources of ecdysteroids. First evidence of this came from oenocytes in larvae of *Tenebrio molitor* (Romer *et al.*, 1974) that were able to synthesise ecdysteroids from cholesterol *in vitro*. It was also suggested that other tissues such as the integument from the last larval instar of *L. migratoria* (Cassier, 1980), pupal wings of *B. mori* (Romer, 1987), and the epidermis of *Tenebrio molitor* (Delachambre *et al.*, 1984; Delbecque, 1990) could be sources of ecdysteroids. In addition, cultures of epidermal cells, isolated from wing pads of *L. migratoria* (Porcheron *et al.*, 1988) secreted ecdysteroids into the medium. These results indicate that the epidermis is able to be a primary source of ecdysteroids while preserving responsiveness to these hormones. During adult reproductive stages, ovarian follicle cells (Hoffmann and Hetru, 1983; Hagedorn, 1985; Smith and Bollenbacher, 1985; Hoffmann *et al.*, 1986; Lanot *et al.*, 1989) and testes (Dumser, 1980; Hoffmann and Behrens, 1982; Loeb *et al.*, 1982; Grimnes and Happ, 1987; Gelman *et al.*, 1989) have been found to be able to synthesise or sequester ecdysteroids.

1.2.2.5.3. Biosynthesis

Ecdysone is biosynthesised from cholesterol and plant sterols (Karlson and Hoffmeister, 1963). Since arthropods are not able to synthesise cholesterol *de novo* (Clayton, 1964), they must obtain it from dietary sources (Karlson and Hoffmeister, 1963). Early steps of ecdysone biosynthesis involve the conversion of cholesterol into 7-dehydrocholesterol. This has been demonstrated in *M. sexta* (Warren *et al.*, 1990) where intact PGs produce 7-dehydrocholesterol from [^{3}H]- and [^{14}C]-cholesterol *in vitro.* The next step is the conversion of 7-dehydrocholesterol through a 3-oxo-Δ^{4}-sterol intermediate (*Schistocerca gregaria,* Davies *et al.*, 1981) into 3β,14α-dihydroxy- 5β-cholest-7-en-6-one (2,22,25-trideoxyecdysone or β-ketodiol) (Rees, 1985). The final reaction in the pathway is the introduction of characteristic structural features of ecdysteroids which involves hydroxylation at C-2, C-22 and C-25 positions. In the PGs and ovarian follicle cells of *L. migratoria,* the order of hydroxylation is C-25, C-22 and C-2 (Hetru *et al.*, 1982). Both 2,22,25-trideoxyecdysone and 22,25-dideoxyedysone are converted into ecdysone. This has also been demonstrated in prepupae of *C. stygia* (Thomson *et al.*, 1971) and in *M. sexta* (Bollenbacher *et al.*, 1977). In *L. migratoria,* not only established ecdysiosynthetic tissues (*i.e.* PGs, ovarian follicle cells) but also Malpighian tubules, fat body and midgut are able to hydroxylate 2-deoxyecdysone into ecdysone (Kappler *et al.*, 1986). This reaction is catalysed by C-2 hydroxylase and localised in the mitochondria (Grieneisen *et al.*, 1993).

1.2.2.5.4. Activation

After being synthesised and secreted from an ecdysiosynthetic tissue, ecdysone is converted into a physiologically active form, 20-hydroxyecdysone which is essential in regulating post-embryonic development (*i.e.* moulting and

metamorphosis) (Gilbert and Goodman, 1981; Fristrom, 1981) and for the reproductive physiology of adult insects, as shown in *A. aegypti* (Fuchs *et al.*, 1979; Hagedorn, 1983). Hydroxylation of ecdysone occurs in tissues peripheral to the PGs such as Malpighian tubules, fat body and midgut (Moriyama *et al.*, 1970; Smith *et al.*, 1983). The process of activation reaction is catalysed by an enzyme system, ecdysone 20-monooxygenase. Ecdysone 20-monooxygenase is a cytochrome P-450 dependent system as demonstrated in *D. melanogaster* (Smith and Mitchell, 1986). This system in the larvae of *D. melanogaster* and in the adults of *A. aegypti* (Smith and Mitchell, 1986) is located in both mitochondria and microsomes.

1.2.2.5.5. Inactivation and storage

Many reactions leading to inactivation of 20-hydroxyecdysone include hydroxylation at C-26, oxidation at C-3, epimerisation at C-3, conversion into ecdysonoic acids, side chain cleavage and conjugation with phosphate, acetic acid, fatty acids or glucose (Koolman and Karlson, 1985; Koolman, 1990).

The formation of these inactivation products may play a significant role in regulating the ecdysteroid titre during insect development and reproduction. This has been observed in several species: *S. peregrina* (Ohtaki *et al.*, 1968), *L. migratoria* (Hoffmann *et al.*, 1974) and in *C. erythrocephala* (Young, 1976; Koolman, 1980) where dramatic variations in the catabolism of radiolabelled ecdysone and 20-hydroxyecdysone were reported during post-embryonic development. Some conjugates (either 22-phophate or 22-acyl ester) may represent storage for excretion or for recycling during further stages of development (*e.g.* in eggs, Bownes *et al.*, 1988; Dübendorfer, 1989). Thus, in concert with biosynthesis, ecdysteroid inactivation provides a fine balance between individual ecdysone and 20-hydroxyecdysone. Such a delicate balance is required for the proper control of development and reproduction (Rees, 1995).

1.3. The roles of ecdysteroids and other hormones throughout insect life cycle

As mentioned above, ecdysteroids are found in every developmental stage: in embryos, larvae, pupae (holometabolous insects) and adults (Rees, 1985, 1989; Delbecque, 1990). Their existence and titres throughout developmental stages indicate that they are synthesised and/or stored in different tissues and suggest that they have distinct stage-specific functions (Redfern, 1989). Tissues response to ecdysteroids varies with both titre and length of exposure. In many stages, exposure to high titre of ecdysteroids for a certain period of time needs to be followed by a low hormone titre period (Schwartz and Truman, 1983). Thus, orderly variations in hormone titres are crucial for temporally organised expression of developmental program (Steel and Vafopoulou, 1989).

1.3.1. Roles of ecdysteroids during embryonic development

Studies on ecdysteroid control of insect embryonic development began when Jones, in 1956, tested ligated embryos of two locust species and concluded that the presumed embryonic endocrine centres had an effect on moulting. Mueller (1963) demonstrated that fragments of embryo were able to moult when incubated with yolk. Boohar and Boocklin (1963) and Boohar (1966) then showed that yolk contains ecdysteroids (reviewed in Hagedorn, 1985). Ohnishi and collaborators (1971) bioassayed the moulting hormone activity of *Bombyx* embryos at various stages, including those from diapausing eggs. They found that the biological activity was high during the developing stages, but relatively low during the diapause period. Several years later a persuasive body of evidence came from investigations in various species including *Oncopeltus fasciatus* (Dorn

and Romer, 1976), *Blaberus craniifer* (Bullière *et al.*, 1979), and *L. migratoria* (Lagueux *et al.*, 1979) showing that ecdysteroids also control embryonic moulting.

In Diptera, a single prominent peak of ecdysteroid titre during embryonic stage of *D. melanogaster* was associated with embryonic cuticulogenesis (Märoy *et al.*, 1988). Wentworth and Roberts (1984) found that deposition and tanning of the cuticle and spiracles in the developing embryos of the flesh fly *S. bullata* were correlated with a peak of ecdysteroid titre.

Other hormones involved in the control of embryonic development

Dorn (1982) showed in *O. fasciatus* that precocene (anti juvenile hormone) treatment on newly laid eggs caused a defect in the secondary ectodermal dorsal closure which could be overcome by application of juvenile hormone. Furthermore, the critical period was recorded a few hours after blastokinesis when the endogenous juvenile hormone titre increased. These findings led Dorn to conclude that endogenous juvenile hormone regulates developmental processes leading to secondary dorsal closure.

In addition to the activity of the juvenile hormones during embryonic stage, PTTH activity has also been detected as early as 25 % embryonic development of *M. sexta* (Dorn *et al.*, 1987) suggesting that differentiation of the L-NSC in this species occurs early during embryonic development. This has led to a speculation that PTTH might have a role in directing embryonic development (*e.g.* embryonic moulting) via stimulation of presumed embryonic PGs to synthesise and release ecdysteroids. Alternative sites of PTTH sources may exist, as indicated in *M. sexta* embryo using the big PTTH monoclonal antibody which immunostains neurones (Westbrook and Bollenbacher, 1990). These authors found that two to four neurones in the frontal ganglion become immunoreactive starting at around 40% embryonic development.

1.3.2. Roles of ecdysteroids during larval development

In larvae, ecdysteroids control a wide range of physiological and biochemical processes involved in development and moulting, including the preparatory steps of the moulting process, such as termination of feeding and the emptying of the gut. During each moulting cycle, hormonal regulation by ecdysteroids involves both stimulation and inhibition of particular processes. Ecdysteroids directly promote early moulting processes: apolysis and proliferation of epidermal cells, secretion of layers of new cuticle, digestion of the old cuticle accompanied by sclerotisation of the new cuticle (reviewed in Riddiford, 1985). While these processes are in progress, the later moulting processes such as production of pigments and ecdysis are inhibited (Truman, 1988).

The role of ecdysteroids in moulting processes have been investigated during larval stages by temporally correlating ecdysteroid titres to developmental events in several insect species. In *B. mori* larvae (*Lepidoptera:* Kiguchi and Agui, 1981) for example, ecdysteroids were involved in spiracle and general body apolysis and the onset of deposition of new cuticle. In *S. bullata* larvae (*Diptera:* Wentworth *et al.*, 1981), ecdysteroids were correlated with apolysis, deposition of tracheal cuticle, pre-ecdysial tanning of spiracles and mouth hooks.

Ecdysteroids also control behavioural events such as the start of the wandering period in the last larval instar. For example, in *M. sexta* (Truman and Riddiford, 1974), a rise in ecdysteroid titre occurs midway through the last larval instar and is referred to as the "commitment peak" (or "wandering peak"). A similar commitment peak is also found in *S. bullata* (Roberts, 1982), at which time larvae commence the wandering behaviour.

At the molecular level, ecdysteroids cause a moult by combining with the ecdysone receptor (EcR) and the ultraspiracle protein (USP) to activate directly a number of regulatory genes whose products both repress ongoing gene expression and stimulate genes associated with the production of the new stage in a cascading fashion (Riddiford, 1993).

Other hormones involved in the control of larval development

During larval stages, PTTHs stimulate the PG to synthesise and secrete ecdysteroids (Goodwin *et al.*, 1978), whereas allatotropins stimulate CA to synthesise JH (Granger *et al.*, 1984). In contrast, PTSHs (Hua and Koolman, 1995) and allatostatin (Duve *et al.*, 1993) inhibit the activity of PG and CA respectively. Stimulation and inhibition mechanisms by which neuropeptide hormones modulate the titre ratio between ecdysteroids and JH, regulate insect post-embryonic development (Hua *et al.*, 1997). High titre of JH leads to a larval/larval moult, while low titre of JH leads to a larval/pupal moult (reviewed by Gäde *et al.*, 1997). Finally, the final stage of a larval moult (*i.e.* ecdysis) is triggered by ETH (Zitnan *et al.*, 1996), and the release of ETH itself is triggered by EH (Truman, 1996).

1.3.3. Roles of ecdysteroids during metamorphosis

During the metamorphosis of holometabolous insects, ecdysteroids promote the differentiation of the imaginal discs (Oberlander and Lynn, 1982) and play an important role in co-ordinating cuticulin deposition and morphogenesis in the imaginal discs (Fristrom and Liebrich, 1986), apolysis and cuticle deposition (Locke, 1990). Ecdysone is also crucial for the degradation of larval fat body and the differentiation of adult fat body (Dübendorfer and Eichenberger, 1985), and

may be involved in triggering changes in gene expression related to reorganisation and differentiation of many other tissues during metamorphosis (Bownes, 1990).

At molecular level, the role of ecdysteroids has been studied in *M. sexta*. During metamorphosis the isoforms involved in the EcR/USP complex change, with the most dramatic switch being the loss of USP-1 and the appearance of USP-2 during the larval and pupal moults. This switch in USP isoforms is mediated by high 20-hydroxyecdysone levels (Hiruma *et al.*, 1999).

Other hormones involved in the control of metamorphosis

Regulation of metamorphosis in insects is performed by the interaction of at least three major hormones: PTTH, ecdysone and JH. When a final instar larva is physiologically ready to undergo metamorphosis, the brain receives messages which provoke the NSCs to synthesise and/or to secrete PTTH (Gilbert *et al.*, 1980). This induces the PG to synthesise ecdysone which is converted to 20-hydroxyecdysone by other tissues (*e.g.* fat body, Malpighian tubules) (Koolman, 1990). An adequate level of 20-hydroxyecdysone is responsible for a cellular re-programming from larval to pupal developmental program (Riddiford, 1976). Nevertheless, this re-programming is not induced while JH is present. Thus, for metamorphosis to occur, inactivation of JH is essential. In *D. melanogaster,* this inactivation is acted by the enzyme JH-esterase (JHE) which is highly selective for JHIII and JHIII bisepoxide (Campbell *et al.*, 1998). The synthesis of JHE is encoded by the structural gene, Est-JH (Rauschenbach and Lukashina, 1983). The JHE activity is regulated by a specific gene system, Ra-JHE (Rauschenbach and Lukashina, 1983). The Ra-JHE gene determines the synthesis or release of the JHE activating factor.

1.3.4. Roles of ecdysteroids in adult insects

1.3.4.1. Ecdysteroids in adult females

The role of ecdysteroids in adult females was first demonstrated in the mosquito *A. aegypti* (Hagedorn and Fallon, 1973). In anautogenous *Aedes* females, vitellogenesis was initiated by a blood meal. Removal of the ovaries after a blood meal inhibited the rate of vitellogenin synthesis by the fat body. The ovaries also induced the fat body to increase the rate of *in vitro* vitellogenin synthesis. Whole-body extracts showed that a peak of ecdysteroid titre was initiated by a blood meal. Ecdysteroid activity was also found in the haemolymph of blood-fed females, but not in that of non-blood-fed females (Hagedorn *et al.*, 1975). The haemolymph ecdysone concentration was equal to the concentration that stimulated a half-maximal response by the fat body of non-blood-fed females *in vitro* (Hagedorn *et al.*, 1975). All these data led to the conclusion that vitellogenin synthesis is controlled by ecdysone from the ovary (Hagedorn, 1985).

Ecdysteroids also control meiotic initiation of the oöcytes as demonstrated in *Locusta migratoria* by Lanot *et al.* (1987). These authors found that the titres of ecdysteroids in the posterior pole, where the oöcyte nucleus is located, was found to be approximately 3 to 4 fold higher than in the anterior pole. A dramatic increase of ecdysteroids was recorded in the posterior pole at the end of vitellogenesis, before chorionation and suggesting a role in meiotic reinitiation and germinal vesicle breakdown. This was then confirmed when oöcytes incubated *in vitro,* did not undergo meiotic reinitiation until exogenous ecdysone was added. This meiotic induction by ecdysone ocurred in a dose-dependent manner (Lanot *et al.*, 1987).

In addition, in higher Diptera, ecdysteroids appear to regulate yolk protein uptake by the ovaries. This is evident in *D. melanogaster* (Richard *et al.*, 1998) from experiments using mutant flies ap^{56f} which have low levels of JH production yet are vitellogenic. Furthermore, breaking a pre-vitellogenic reproductive diapause in these mutants, by increasing temperature, increased the rate of ecdysteroid synthesis by the ovaries. Meanwhile JH production remained at a low level. This resulted in vitellogenic oocytes. This is further supported by the fact that pre-vitellogenic diapause was also terminated by injection of 20-hydroxyecdysone alone into the female abdomens.

Other hormones involved in the control of female reproductive cycle

In Diptera, ESG is also required to stimulate ovaries to produce ecdysone (Adam *et al.*, 1997). Although JH does not appear to have a significant role in yolk protein uptake in Diptera, it is essensial to enhance ovarian ecdysteroid production in *D. melanogaster* (Schwartz *et al.*, 1985), *M. domestica* (Agui *et al.*, 1995), and *P. regina* (Qin *et al.*, 1995), and to condition the fat body to respond to ecdysone in *A. aegypti* (Flanagan and Hagedorn, 1977), in *P. regina* (Qin *et al.*, 1995). JH is also necessary to prime follicular cells to respond to nutritive diet and to prime the ovaries to become responsive to ESG in *M. domestica* (Adam *et al.*, 1997) and in *P. regina* (Yin *et al.*, 1994).

1.3.4.2. Ecdysteroids in adult males

The role of ecdysteroids in adult males appeared to be associated with spermatogenesis. For example, the rate of cell division in the spermatogonial cells of *R. prolixus* (Dumser and Davey, 1974) is promoted by the presence of 20-hydroxyecdysone. Schmidt and Williams (1953) found that the haemolymph of developing *B. mori* contained a macromolecular factor that induced

spermatogenesis *in vitro*. The entry of this macromolecular factor was stimulated by 20-hydroxyecdysone (Kambysellis and Williams, 1971). In *G. bimaculatus*, high ecdysteroid titres are associated with the growth of male accessory organs and spermatophore production (Hoffmann and Wagemann, 1994). In Diptera, although the presence of ecdysteroids in adult males has been shown in the testes of *C. vicina* (Koolman *et al.*, 1979), their function is still not clear.

Other hormones involved in the growth of male reproductive system

Secretion of ecdysteroids by the testis was shown to be induced *in vitro* by a brain peptide called testis ecdysiotropin (TE) in *Heliothis virescens* (Loeb *et al.*, 1982) and in *Lymantria dispar* (Loeb *et al.*, 1988). This peptide was reported to be different from PTTH in several physical properties and its requirement for exogenous 20-hydroxyecdysone (Loeb *et al.*, 1986). JH also appears to be involved in the regulation of growth and protein synthesis in the accessory reproductive gland of male *Locusta migratoria* (Braun and Wyatt, 1995). It was shown that following inhibition of endogenous JH with ethoxyprecocene, the accessory gland failed to grow but growth was restored by a single application of the JH analog, pyriproxyfen.

In summary, ecdysteroids are present in all developmental stages. In co-operation with other hormones: PTTH, EH, ESG, as well as JH, ecdysteroids play crucial roles in regulating growth and development throughout the insect life cycle in a stage- and tissue-specific manner. To begin to understand the mechanisms of hormonal control of growth and development, measurement of hormonal concentration in every stage of development is mandatory.

1.4. Previous studies of *Lucilia cuprina*

The Australian sheep blowfly, *L. cuprina,* is well known as one of several species responsible for the cutaneous myasis of sheep, also called "blowfly strike", which causes annual loss of millions of dollars to the Australian sheep industry. About 80 % of primary strikes are caused by *L. cuprina* (CSIRO, 1992). Understandably, *L. cuprina* has been studied by several research groups mostly on aspects of their ecology, development, reproduction, behaviour and insecticide resistance. The growing awareness of the drawback, both financially and environmentally, of conventional pest control programmes *e.g.* chemical jetting, has renewed interest in the endocrinology of *L. cuprina* which could provide invaluable data for future application in pest management.

There are indeed very few data published to date concerning the hormonal aspects of growth and development in *L. cuprina*. No systematic investigation of hormonal levels throughout the entire development exists in *L. cuprina*. Only one study by Barritt and Birt (1970) using a bioassay measured the concentration of prothoracic gland hormone during metamorphosis of *L. cuprina*. More recently, ecdysteroid titres in the ovaries have been determined using RIA in adult *L. cuprina* (Clissold *et al.*, 1993). At the molecular level, Hannan and Hill (1997) cloned and characterised LcEcR- a functional ecdysone receptor from *L. cuprina* larvae.

Juvenile hormone, on the other hand, has received relatively more attention in *L. cuprina* in recent years. The novel JHB_3 released *in vitro* by the ring glands of late third instar larvae and by the CA of the adults was identified and quantified by Lefevere *et al.* (1993). Furthermore, Sutherland and East (1997) investigated the regulation of JHB_3 synthesis by ring gland complexes

from third-instar larvae *in vitro*. Hormone synthesis is regulated by three distinct mechanisms: rapid decline in hormone release, a neurally mediated inhibition by the brain, and a neural inhibition. These mechanisms were then verified by East *et al.* (1997) with the evidence that ring glands and brain-ring gland complexes from third instar larvae released more JHB_3 than comparable preparations from pre-pupae, while isolated CA segments of the gland were more active than intact brain-gland complexes. The role of JH in reproduction, on the other hand, was investigated as early as 1972, when Clift studying the activity of CA based on histological data during reproductive development, suggested that a protein meal may induce JH biosynthesis. This suggestion was only verified much later, when Clissold *et al.* (1993) measured the rate of JH biosynthesis *in vitro* in isolated CA-CC complexes during the ovarian cycle.

1.5. Aims

This study aims to investigate the steroidal hormonal control of growth and development throughout the life cycle of the blowfly *L. cuprina*. This objective is approached by analytical measurements of ecdysteroid titres and examination of temporally related developmental events from the embryonic stage through to the adult reproductive stage. For this purpose, accurate staging methods are developed and standardised. The effect of protein nutrition on the adult flies is also investigated.

Chapter 2

MATERIALS AND METHODS

2.1. Breeding of insects

Wild-type *L. cuprina* (Diptera, Calliphoridae) were obtained from the Division of Entomology, CSIRO Canberra. Throughout the whole life cycle, the flies were maintained in a constant temperature room of $27^{\circ} \pm 1^{\circ}$ C with 40 - 50 % relative humidity, under a 12 : 12 light/dark cycle with "lights on" at 0600 h local time. Adults were reared from eclosion (day 1) in 30 cm square wire framed cages covered with fine 1 mm mesh cloth (Fig. 2). Flies were continuously fed with dry sugar cubes (CSR Australia) and given *ad libitum* access to sliced fresh lamb liver in 10 cm petri dishes, as a source of protein for 48 h starting at 0900 h on day 2 (Smith, 1985). Water was constantly supplied by placing an inverted jar over a petri dish containing fine plastic wire mesh.

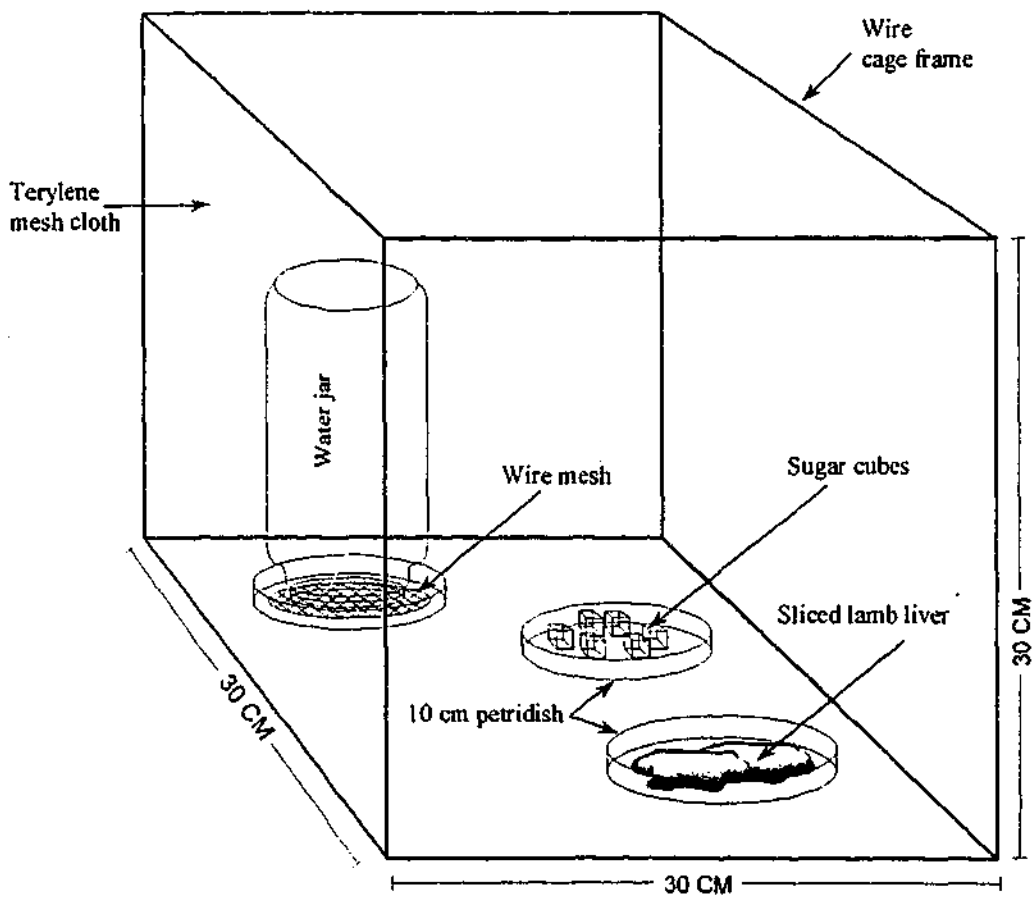


Fig. 2. A cage used for breeding adult *L. cuprina*

Eggs were collected from oviparous gravid adult flies on the fifth day after adult eclosion (considering the day of eclosion as day 1), by placing sliced fresh lamb liver for 1 h (0700 - 0800 h) in a cage where the flies were maintained. The eggs were then transferred to an aluminium foil boat of minced fresh liver with the initial density of 28 mg of eggs/100 g medium (Smith *et al.*, 1981). Each boat consisted of about 400 g minced lamb liver. The boat was placed in a non airtight plastic container 25 cm in diameter and 10 cm high containing a 2 cm thick layer of vermiculite (Neuchatel, Victoria) in its bottom. The eggs hatched 11 - 12 h following oviposition and the larvae remained on the same medium until the end of the larval feeding stage.

Following voracious feeding during the early instars, larvae started leaving the food in the scotophase of day 3 of development (c. 96 h after hatching) and entered the vermiculite to pupariate. Pupariation occurred at c. 128 h from hatching. Pupae were separated from the vermiculite by using a coarse plastic mesh sieve and transferred to plastic petri dishes with perforated lids to provide air circulation. Five days following pupariation pupae were transferred into net enclosed breeding cages as previously described.

Adult flies emerge from their puparia over a two-day period starting from midnight of the sixth day after pupariation. Flies which emerged on the first day were predominantly males and were discarded. Only those which emerged during a two hour period (0500 and 0700 h) on the second day of emergence were used for experiments. Adults were reared as previously described.

2.2. Ageing of the insects

2.2.1. Eggs

In order to synchronise or at least to minimise the variation of the starting time of embryonic development, the period in which eggs were taken from gravid adult females on the fifth day after eclosion was set to a maximum period of 1 h (from 0700 to 0800 h). After landing on the sliced lamb liver provided for oviposition, gravid adult females took about 15 - 30 min before commencing laying eggs. In a closely related species *L. sericata,* the egg undergoes its first maturation division 5 - 10 min following oviposition (Davis, 1967). Considering this finding, the starting time of embryonic development was taken as 0730 h and designated as 0 h of age.

2.2.2. Larvae

2.2.2.1. Determination of larval instars

In all cases larval development was aged from the time of eclosion from the egg. This time was designated as 0 h of larval age. The determination of the developmental stages follows that of Hinton (1973) which is mainly based on distinct morphological and structural changes of the integument.

The structures of the cephalopharyngeal apparatus, especially the mouth hooks, and also the morphology of the posterior spiracles, have been used as convenient and reliable indicators of the instars of larval flies (Weismann, 1864). It has been proven that the rigid cuticular structures of the mouth hooks and posterior spiracles change shape and size only at each moult in the fleshflies *T. impatiens* (Roberts, 1976) and *N. bullata* (Wentworth *et al.*, 1981).

In order to determine the start and the end of each stage of larval development (instar) in *L. cuprina*, the morphology and the size of mouth hooks were examined. Samples were taken at two hour intervals from the same population as that used for experimental purposes and placed in Carnoy's fixative (Pantin, 1960) and stored for further histological processing. The anterior segments were dissected free, boiled in 5% KOH for 5 min, dehydrated in 100% ethanol, cleared in [illegible] and mounted on glass slides in Canada Balsam. The preparations were then examined under a Leitz Orthoplan microscope fitted with interference contrast optics and the functional mouth hooks were measured using an eyepiece 16 [illegible] screw micrometer. The length of a mouth hook was taken as the distance between the tip of the hook and the posteriodorsal process. The degree of sclerotisation of developing mouth hooks was examined and scored using criteria developed by Roberts (1976). A larval population was considered to have undergone moulting when the majority of the larvae had developed new mouth hooks.

2.2.2.2. Formation of the PFL (post-feeding larval stage)

A third instar larva which has irreversibly ceased feeding is defined as a PFL (Fraenkel and Bhaskaran, 1973). Estimations of the time of larval exodus from the food *i.e.* the entrance to the PFL were determined by seeding thirteen liver boats as previously described with eggs taken from gravid adult females at three different times. The first five liver boats were seeded with eggs laid at 0700 - 0800 (1 - 2 h following lights on). The second five boats and the remaining three boats were seeded with eggs laid at 1430 - 1530 (8.5 - 9.5 h following lights on) and at 2330 - 2430 (9.5 - 10.5 h following lights on) respectively (Smith, *et al.*, 1981). From preliminary observations, it was found that larvae started leaving

the food during the night of day 3 following larval hatching. Considering this fact, each boat was checked every hour from the third day of larval development. Larvae which had left the food were counted and discarded. This was done with care to minimise any disturbance which could cause the feeding larvae to delay their commencement of PFL (Thompson *et al.*, 1970). During the scotophase the cultures were examined under a dim red light using a Kodak Wratten 92 filter with a 25 W bulb to prevent any disturbance of the photoperiodic factor which may influence the start of the PFL (Chapman, 1969).

2.2.3. Prepupae, Pupae and Pharate Adults

During prepupal, pupal and pharate adult stages of development, the insects were staged using the criteria developed for cyclorraphous flies by Fraenkel and Bhaskaran (1973). For this purpose, during the last hours of the PFL stage the cultures were checked every 15 min. Larvae that had contracted and were completely immobile were considered to have entered the white prepupal stage (WPP). They were removed from the vermiculite and transferred to petri dishes and aged as 0 h of prepupal, pupal and pharate adult stages of development.

For accurate staging of prepupal, pupal and pharate-adult stages of development, the animals were examined histologically. Specimens from appropriate ages were punctured with a fine needle and placed into alcoholic Bouins' fixative (Pantin, 1960, Appendix 1) for 24 h. Specimens were then washed in 70% ethanol until no yellow colour appeared and dehydrated in a series of gradual increases by 10% of ethanol concentrations from 70% to 100%, 6 h for each step. The specimens were then put into a series of gradual LR-White resin (The London Resin Co. Ltd.) in ethanol from 10% to 100% by 10% increases for

6 h per step. The specimens were then polymerised under UV light and nitrogen flow in order to harden the resin. Sagittal sections of the specimens were made by using an OMU3 microtome (Reichert, Austria) to a thickness of 10 µm and stained with 3% Toluidine blue (pH=9). The preparations were then examined under a Leitz Orthoplan microscope with interference contrast optics.

In order to find out whether it is possible to sex individuals before the adult stage, WPP (0 h) were weighed individually using a Mettler HK 60 balance (Switzerland), throughout the stage. Each individual was maintained separately until adult eclosion, at which time the animals were accurately sexed. Statistical analysis was then performed to compare male and female weights.

2.2.4. Adults

Adult flies were aged from the time of eclosion. In order to obtain a more synchronised population and balanced ratio between males and females during the adult development, flies emerging on the first day were discarded. All flies emerging over a two-hour period (0500 - 0700 h) on the second day of emergence after lights on were collected for the experimental purposes and considered to be the same age and designated as 0 h of development (Vogt *et al.*, 1974).

The determination of the developmental stages of the adult females of *L. cuprina* is based upon the reproductive cycle and follows the 6 stage criteria (Stages 0 - V) developed by Vogt *et al.* (1974). This classification is based upon the system developed for *Hippelates collusor* (Adams and Mulla, 1967) and modified for *L. cuprina* (Clift, 1972).

2.3. Preparation of the living material for radioimmunoassay

2.3.1. Eggs

Eggs were collected from the liver every 2 h from oviposition until hatching. They were weighed with a digital electronic balance (Mettler HK 60, Switzerland) and counted. Each replicate consisted of 6.13 - 12.35 mg of about 114 - 230 eggs. Samples were transferred to 1.5 ml polypropylene Eppendorf tubes (Eppendorf Geratebau, Hamburg, Germany) for ecdysteroid extraction (described in section 2.4.2).

2.3.2. Larvae

During the first and second instar, larvae were sampled in groups for each replicate due to the small size; whereas in the third instar, the larvae were large enough to be sampled individually. Every 2 to 4 h starting from the time of hatching, the larvae were collected in 10 cm petri dishes from the rearing medium, washed in water to free them from liver and dried on tissue paper. To facilitate handling the larvae were immobilised by placing the petri dishes on ice. The larvae were weighed using a digital electronic balance (Mettler HK 60, Switzerland) and counted. Larvae were then processed in the same way as the eggs, as described in section 2.3.1..

2.3.3. Prepupae, pupae and pharate adults

Starting from the time of pupariation until adult eclosion, female and male flies were sampled individually at four-hour intervals for ecdysteroid extraction. The animals were then processed in the same way as for the egg and larval stages, as described in section 2.3.1.

2.3.4. Adults

Adult flies were sampled every 4 h starting from the time of eclosion and immobilised using CO_2 gas (CIG). Female and male flies were sampled individually, weighed and processed as in section 2.3.1.

2.3.5. Female reproductive system

Starting from 48 h after emergence adult females were immobilised using CO_2 and the reproductive system (ovaries, oviduct and associated organs) dissected out under 0.15 M NaCl (Barton-Browne *et al.*, 1986) every 4 h. The rest of the body was assayed separately and processed in the same way as in section 2.3.1.

2.4. The radioimmunoassay (RIA) for ecdysteroids

2.4.1. Basic principles of method

Measurements of ecdysteroids in most bioassays used previously have required relatively large amount of materials, and are expensive and time consuming. The method used here was a competitive binding assay, first introduced by Borst and O'Connor (1972a,b). This method enables ecdysteroid levels to be determined in tissues of a single insect.

This assay includes three components: (i) a *binding protein* (A-2 rabbit ecdysone antiserum) which was kindly provided by Dr. Bollenbacher, Department of Biology University of North Carolina, Chapel Hill, NC, USA, (ii) *ligands* (ecdysteroids) which were the materials being analysed, and (iii) a *labelled ligand* (^{3}H-ecdysone) (New England Nuclear, Du-Pont, Boston, Mass, USA), used as a tracer. When these three major components are mixed together, a competition occurs between the ecdysteroids present in the samples and the ^{3}H-ecdysone for a limited number of binding sites available in the antiserum. As the concentration of the ecdysteroids in the sample increases, the amount of the ^{3}H-ecdysone bound to the antiserum in the assay decreases. After the formation of the complexes between ecdysteroids, ^{3}H-ecdysone and the antiserum, the complexes are coagulated and separated from the free ecdysteroids and ^{3}H-ecdysone. The amount of ^{3}H-ecdysone (degree of radioactivity) in the complexes (or bound fraction) is then counted and termed RIA response expressed in cpm. A standard curve is made with a series of known quantities of 20-hydroxyecdysone as the ligand. By plotting the degree of radioactivity in an unknown sample to the standard curve, the quantity of ecdysteroids in the sample is estimated.

2.4.2. Sample preparation for crude ecdysteroid extract

For ecdysteroid extraction, the animals from every developmental stage were sampled at specified times (described in details in section 2.3.) and homogenised in a 1.5 ml polypropylene Eppendorf tube containing 200 µl of 100% analytical grade methanol (Anala, BDH Chemicals, Victoria, Australia) for about 3 to 5 min using an electric motor driven Teflon pestle (Citenco, England). The homogenate was centrifuged at room temperature for 10 min at 2000 rpm in a bench top centrifuge (Roto Uni, Germany). Duplicate samples of 50 µl of the ecdysteroid extract (supernatant) of each replicate were taken and transferred to 6 x 50 mm culture tubes (Kimble Owens, Ill, U.S.A.) and dried down under vacuum. Samples were covered with parafilm and stored at 4° C until further processing.

Preliminary assays showed that some samples from the third instar larvae and liver-fed adult females contained a high proportion of lipid as indicated by the floating of the coagulated ecdysteroid-antiserum complexes that avoided separation of the ecdysteroid-antiserum complexes from the free fraction. In order to remove the non-polar lipids the samples were partitioned by adding 200 µl of a chloroform:methanol (1:1, v/v) solution as well as 80 µl water to the dried ecdysteroid extract. The samples were mixed using a Vortex Genie (Scientific Industries, Springfield, Mass., USA) for 3 min and centrifuged at 3000 rpm for 20 min at 4° C in a Sorvall RT 6000 refrigerated centrifuge (Norwalk Conn., USA). The aqueous methanol phase was transferred to a 6 x 50 mm Kimble tube and dried down under vacuum. Samples were covered with parafilm and stored at 4° C until further processing. The procedure for ecdysteroid extraction is summarised in Fig. 3, A.

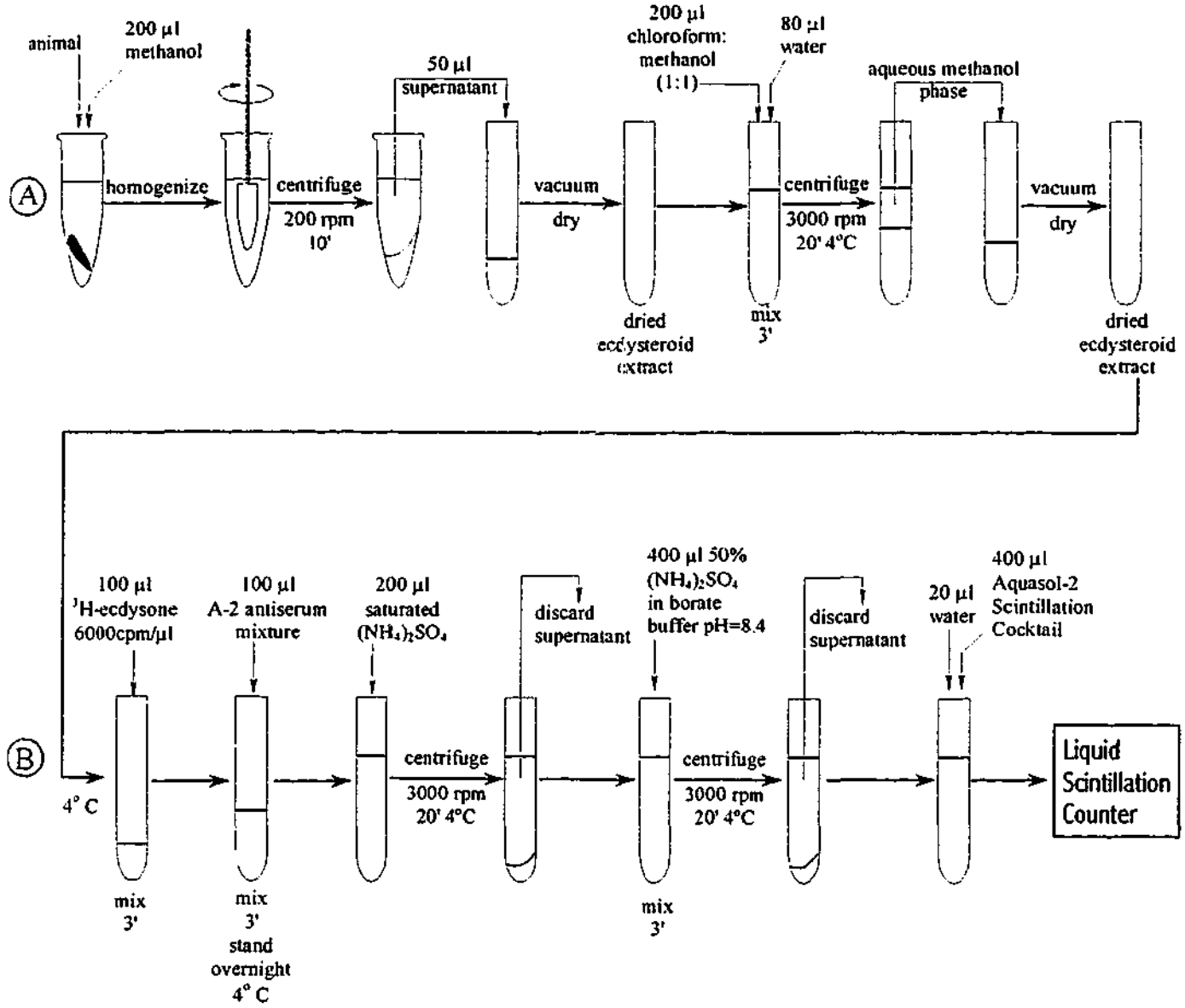


Fig. 3. A flow diagram of the procedure for ecdysteroid extraction (A) and the radioimmunoassay (B).

2.4.3. Antiserum binding capacity test

In order to obtain the appropriate binding capacity for the assay, the A-2 rabbit ecdysone antiserum (22-succinyl derivative) which has specific affinities to ecdysone, 20–hydroxyecdysone, inokosterone and ponasterone (Bollenbacher, pers. Comm.) needs to be tested. For this purpose, 100 µl of tracer ligand (^{3}H-ecdysone) of 80 cpm/µl was incubated with 100 µl of serial doubling dilutions of the antiserum from 1.6 to 0.0015 %, v/v in borate buffer (pH=8.4). The amount

of radioactivity in the bound fraction between ^{3}H-ecdysone and the antiserum for each concentration is compared to the total count of 100 µl ^{3}H-ecdysone in the absence of antiserum and expressed in %. The curve is shown in Fig. 4. The concentration of the antiserum chosen for use in the radioimmunoassay was 0.4% which lies on the steep area (Chard, 1990) and was sufficient to bind approximately 42% of ^{3}H-ecdysone.

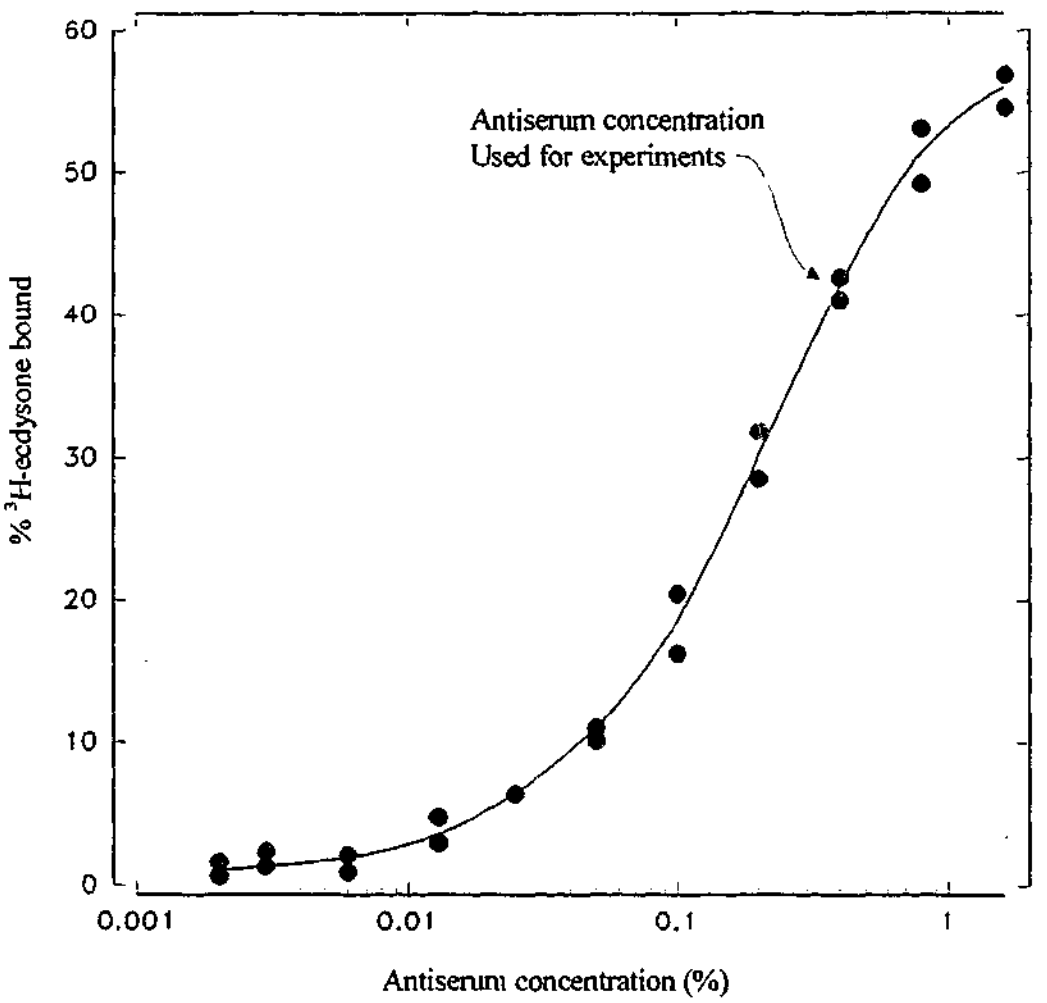


Fig. 4. An A-2 rabbit ecdysone antiserum dilution curve. The antiserum was diluted in borate buffer (pH=8.4) with doubly increasing concentrations from 0.002 to 1.6 % (v/v). One hundred µl of each dilution was incubated with 100 µl of tracer ligand (^{3}H-ecdysone) of 80 cpm/µl. The bound fraction was counted in an LKB Scintillation Counter. The concentration of the antiserum chosen for use in the radioimmunoassay was 0.4% and is sufficient to bind approximately 42% of ^{3}H-ecdysone.

2.4.4. Preparation of standard curves

Each RIA standard consisted of: (i) a series of ten tubes containing different quantities of unlabelled ecdysone, (ii) the background tubes, and (iii) the reference tubes. All tubes were prepared in two replicates. The background samples consisted of tubes containing 100 µl ^{3}H-ecdysone and 100 µl bovine serum albumin. These samples are used for correcting the error generated from the impossibility of total elimination of the unbound fraction of ^{3}H-ecdysone from the sample tubes. The reference samples consisted of tubes containing 100 µl ^{3}H-ecdysone and 100 µl A-2 antiserum. These samples are used for calculating the RIA response in the absence of unlabelled ecdysone.

Preliminary counts were performed in order to obtain a suitable standard range in which the values of ecdysteroid concentration calculated in the samples would fall in the linear part or the steepest slope of the standard curve. This is the most sensitive area of the standard curve in which relatively small changes in the concentration of ecdysteroids in samples produce a significant shift in the RIA response. Thus the assay selected for embryonic, larval, prepupal, pupal and pharate adult stages used standard samples with a range of ecdysone quantity of 0, 0.05, 0.1, 0.25, 0.5, 1, 2, 4, 8 and 16 ng. This was termed the micro-assay (Fig. 5). The assay for adult flies used standard samples with a range of ecdysone quantity of 0, 7.8, 15.6, 31.25, 62.5, 125, 250, 500, 1000 and 2000 pg. This assay was termed micro-micro-assay (Fig. 6).

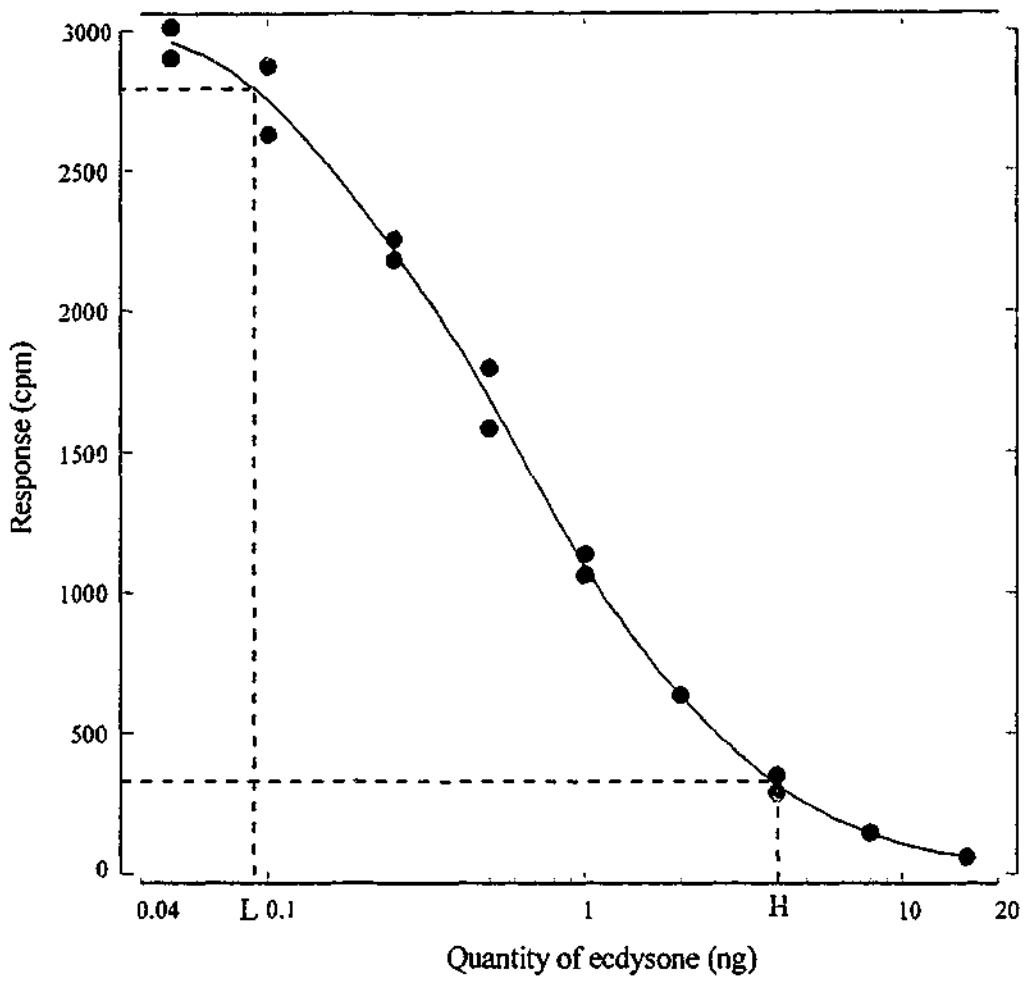


Fig. 5. Standard curve for a micro assay used for all developmental stages except the adults. The line (curve) represents the best-fitted values of two replicates indicated by solid circles (●). Where variation is small, the two replicates are shown as one. L= the lowest; H= the highest ecdysteroid concentration measured in the unknown samples.

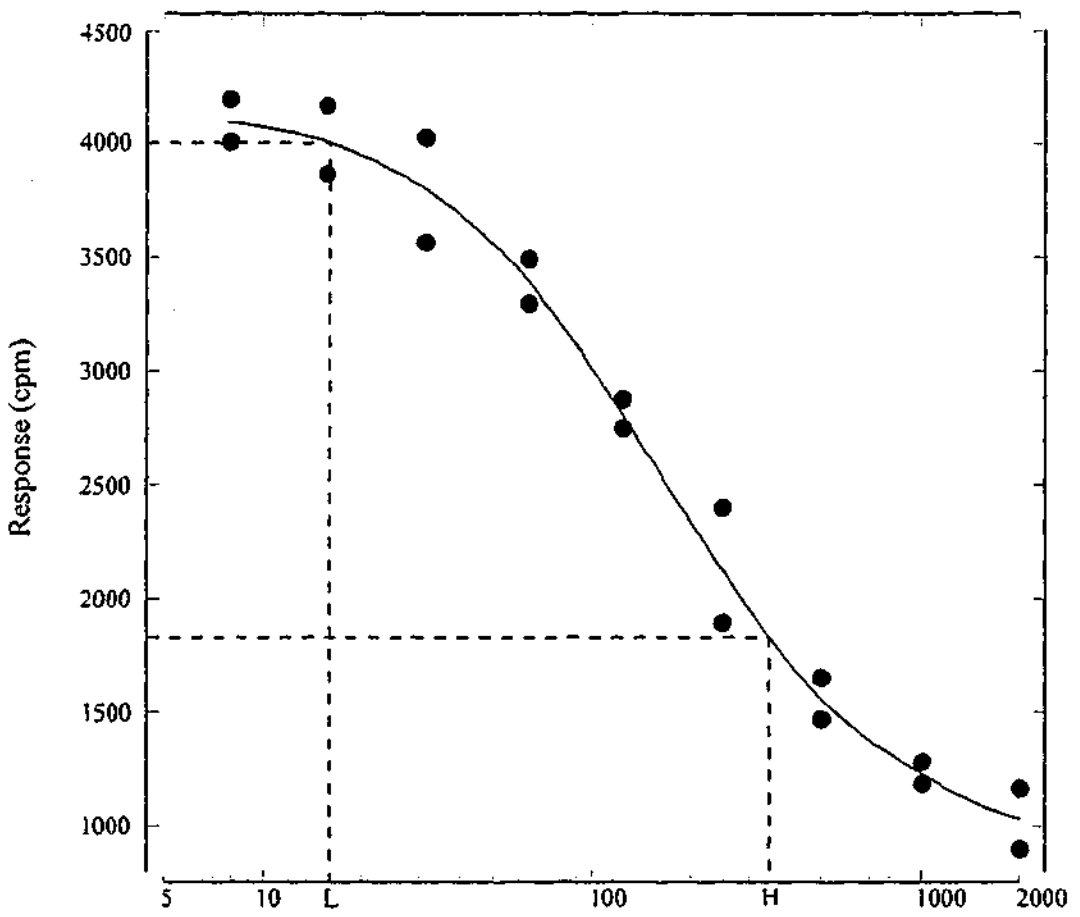


Fig. 6. Standard curve for a micro-micro assay used for adult flies. The curve (line) represents the best-fitted values of two replicates indicated by solid circles (●). L= the lowest; H= the highest ecdysteroid concentration measured in the unknown samples.

2.4.5. The assay

The procedure of the radioimmunoassay is summarised in Fig. 3, B. Firstly the standard and unknown samples were resuspended by adding 100 µl of ^{3}H-ecdysone solution (80 cpm/µl) in borate buffer (pH=8.4) (Appendix 1). This solution was added to the dried ecdysteroid extract at 4° C and mixed thoroughly using a Vortex Genie (Scientific Industries, Mass., USA) for 3 min. One hundred microlitre of 0.4% A-2 antiserum solution in borate buffer (Appendix 1) was added to the sample tubes except the background tubes. The samples were vortexed again and then incubated overnight at 4° C. To facilitate separation of the antiserum-ligand complexes from the unbound fraction, the complexes were coagulated by adding 200 µl of saturated ammonium sulphate $(NH_4)_2SO_4$ to the sample tubes at 4° C. The samples, after being left to stand for 20 min at 4° C, were vortexed and centrifuged at 3000 rpm using a Sorvall RT 6000 refrigerated centrifuge for 20 minutes at 4° C. The supernatant (unbound fraction) was withdrawn from the sample tubes and discarded by using a clean Pasteur pipette connected to a vacuum line. The samples were washed a second time by adding 400 µl 50% $(NH_4)_2SO_4$ in borate buffer at 4° C, and then vortexed until the precipitate was completely resuspended. Finally, following centrifugation at 3000 rpm for 20 min at 4° C, the supernatant was discarded as described above. Twenty microlitre of distilled water was added to each sample tube to dissolve the precipitate before addition of 400 µl of Aquasol-2 scintillation cocktail (Du Pont, Boston, Mass., USA). The radioactivity was counted in a computer-controlled LKB Wallac 1214 Rackbeta Liquid Scintillation Counter after being allowed to stand in the machine for 24 hours in order to reduce chemoluminescence. Each

sample was counted for 5 minutes. The software used was Wallac Rackbeta Scintillation Counter Programme. Standard curves and counting results were calculated, plotted and printed automatically.

2.5. Statistical analysis

Several methods of statistical analysis were applied to help interpreting, inferring and presenting data collected from the experiments. All of the calculation of the data analysis was performed using a statistical and graphical software, Systat and Sygraph version 5.0 for DOS.

A two-sample t-Test was used to compare the mean body weight of 0 h female and male white prepupae. During the later stages of development (prepupal, pupal, pharate adults and adult stages) a paired two-sample t-Test for means was employed to compare the mean body weights of both sexes and ecdysteroid titres in the females and males of many pairs of selected ages. The null hypotheses (H_o) are that $\mu_1=\mu_2$ *i.e.* the mean body weight in the females is the same as that of the males, and the ecdysteroid titres in females and males are the same.

A simple linear regression analysis was used to measure the trend of weights in relation to ages throughout the development of prepupae, pupae, pharate adults and adults.

To infer the data (weights, ecdysteroid titres) collected from different ages at each developmental stage (embryonic, larval, prepupal, pupal, pharate adult and adult), multiple comparisons with one-way analysis of variance were performed. This method enables comparisons of multiple means, locating the difference among the population means and testing the degree of significance of the

difference between any possible pair of population means. The null hypothesis (H_o) is that $\mu_1=\mu_2=\mu_3=\mu_4=\mu_5.....=\mu_\kappa$, where μ is the mean and κ is the number of means compared. When the analysis of variance revealed significant differences between means, the Tukey-hypothesis test was performed to find out where the differences were located among the k population means. This test also measures the degree of significance of the difference between each pair of means with a confidence interval of 95%. The degree of significance is expressed as a probability within a range of values from 0 - 1. If the probability level is < 0.05 the null hypothesis (H_o) is rejected.

Chapter 3

RESULTS

Introduction

To study the roles of ecdysteroid hormones of *L. cuprina,* developmental events were examined at both macroscopic and microscopic levels and the titre of ecdysteroids was quantified by RIA throughout the life cycle. Table 1 summarises the developmental events observed throughout the life cycle. In the following sections the hypothesis that ecdysteroids play important roles in regulating specific developmental events is tested.

Table 1. A summary of developmental events of *Lucilia cuprina* reared at 27° C, under 12D:12L cycles and 40-45% RH, following the terminology of Hinton (1973) and Wentworth (1982).

Age (h)				Stage of development	Developmental event
From Oviposition	From Hatching	From Pupariation	From Adult Eclosion		
0					Oviposition
				Embryonic	
12	0				Hatching
				Instar I	
	18				Moult I
				Instar II	
	34				Moult II
				Instar III	
	95-104				Leaving Food
				Post-feeding	
	128	0		White Prepupa	Pupariation
				Prepupa	
		8			Larval/Pupal Apolysis
				Crypto-cephalic	
		24			Evagination of Head
				Pupa	
				Phanero-cephalic	
		40			Pupal/Adult Apolysis
				Pharate Adult	
		160-184	0		Adult Eclosion
				Adult	
			97		Oviposition

3.1. Embryonic stage

3.1.1. Aspects of embryonic development

As shown in Fig. 7, freshly laid eggs of *L. cuprina* are elongated, white and slightly curved towards the dorsal aspect, weighing 0.065 ± 0.008 mg (± SEM, n=5) with a length of approximately 1400 μm. The egg is covered with an opaque chorion with a hatching pleat on its dorsal side (CH and HP, Fig. 7). Underneath the chorion is the vitelline membrane which encloses the egg (VM, Fig. 7). Since the vitelline membrane is transparent, the developing embryo can be observed in whole mount under a light microscope after removing the chorion. Newly laid eggs showed a white opaque diffused yolk mass inside (Y, Fig. 7). Segmentation of the embryo was observed at 7 h following oviposition (Fig. 8). The tracheal trunks started to be visible two hours later (Fig. 9). This event was followed by the tanning of the posterior spiracles and the appearance of the gut (Fig. 10) and embryonic movements observed at 11 to 12 h (Fig. 11). By 12 to 13 h following oviposition, larvae started to hatch. Detailed developmental events observed during the embryonic stage are summarised in Table 2.

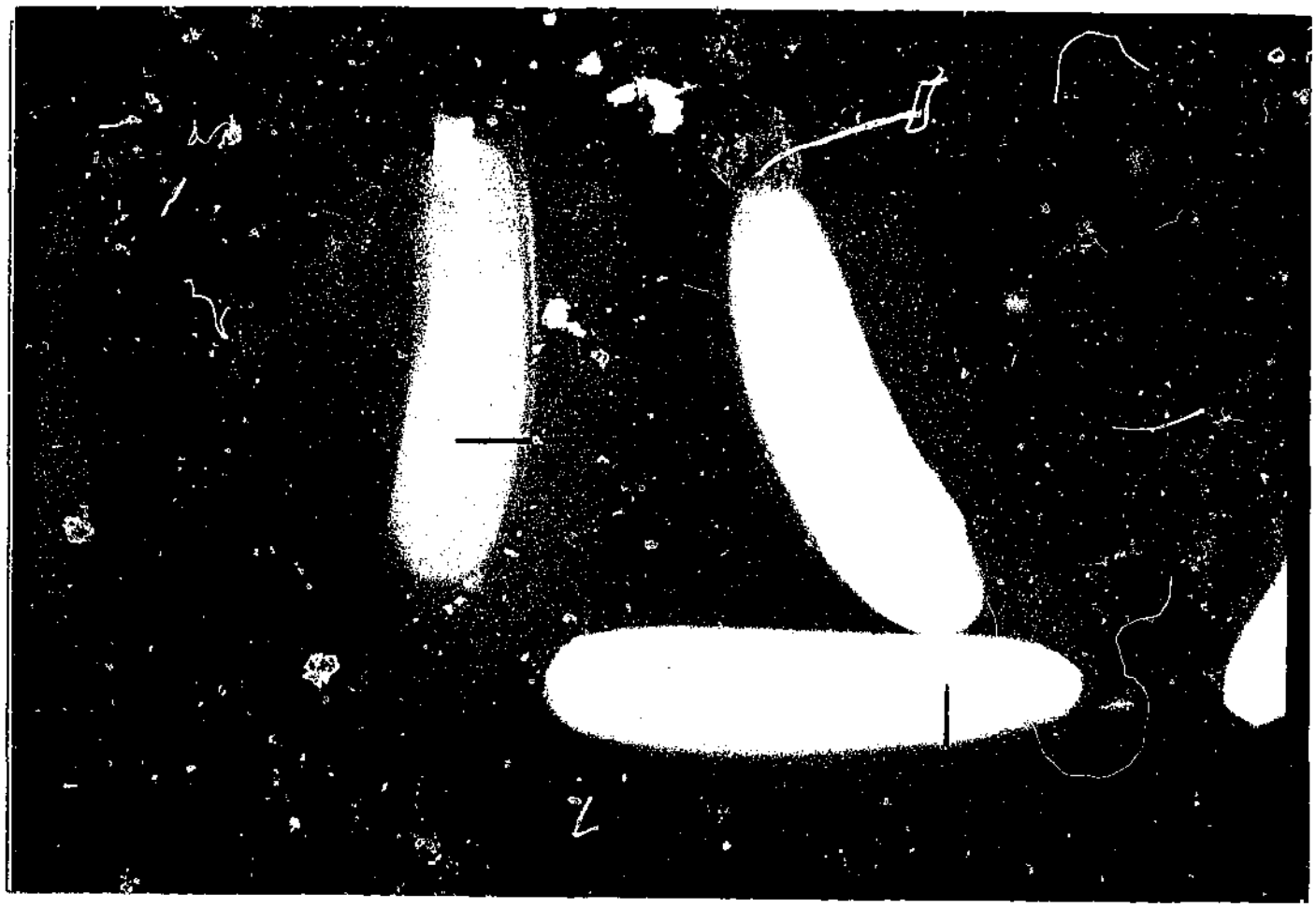


Fig. 7. Dechorionated (left) and intact (right) newly laid eggs of *Lucilia cuprina* viewed under a Nikon Stereomicroscope with GB12 green back filter. VM, vitelline membrane; Y, yolk; CH, chorion; HP, hatching pleat.

400 µm

Fig. 8. Dechorionated eggs of *Lucilia cuprina* at 6 – 7 h after oviposition showing segmentation of the embryo. VM, vitelline membrane. Photographic conditions as in Fig. 7.

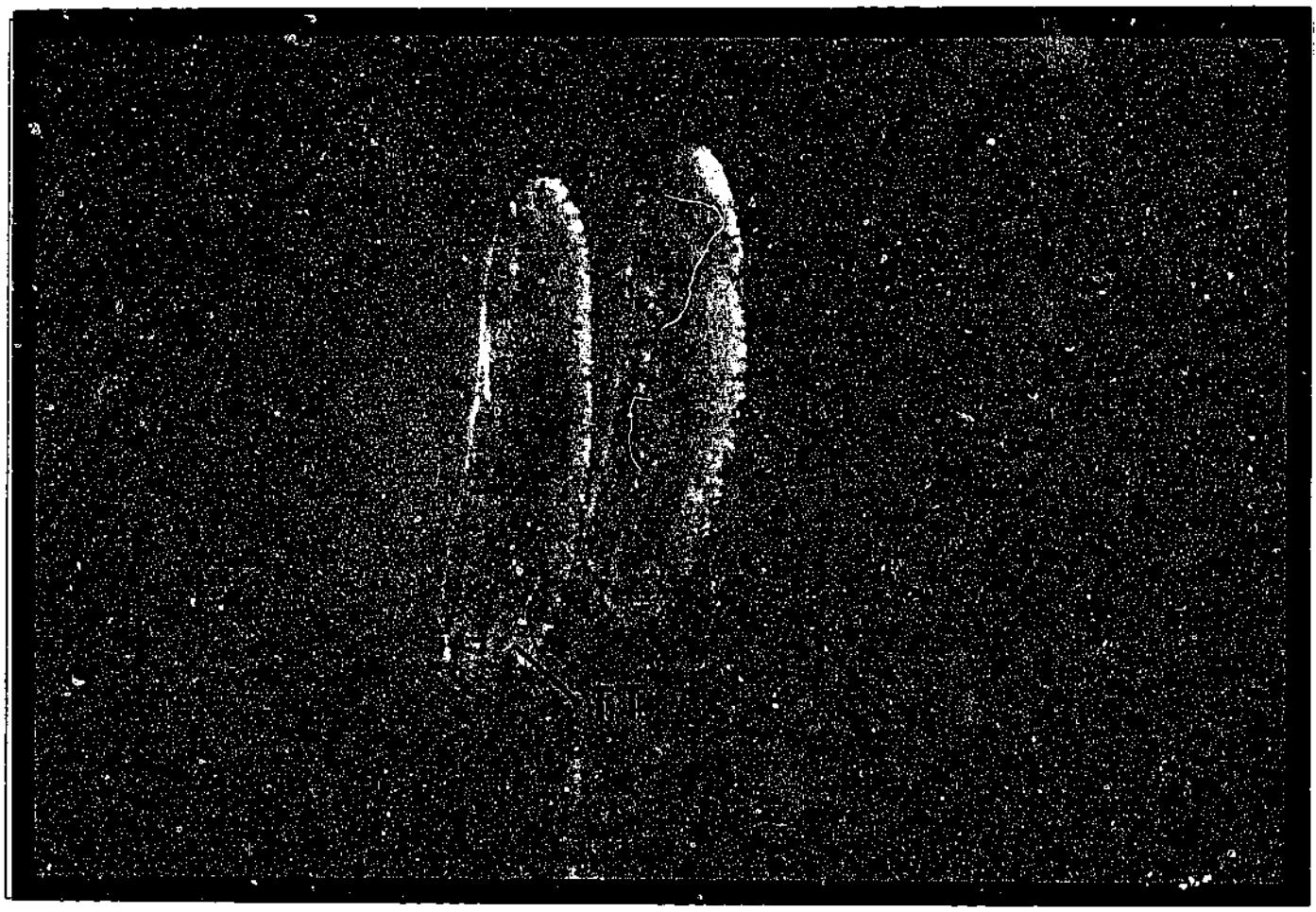


Fig. 9. Dechorionated eggs of *Lucilia cuprina* showing the embryonic tracheal trunks (TT) at 8 - 9 h following oviposition. Photographic conditions as for Fig. 7.

Fig. 10. Dechorionated embryo of *Lucilia cuprina* at 10 - 11 h after oviposition showing the tanning of tracheal trunks and posterior spiracles (PS) and the appearance of the gut (G). Photographic conditions as for Fig. 7.

Fig. 11. A dechorionated egg of *Lucilia cuprina* showing a mature embryo at 12 - 13 h after oviposition just about to hatch. Photographic conditions as for Fig. 7.

3.1.2. Endocrinological aspect

Newly laid eggs were sampled at 2 h intervals over a period of 12 h until hatching, *i.e.* ecdysteroids were extracted for RIA as previously described. Statistical analysis of the results shown in Fig. 12 is presented in Appendix 2.

In newly laid eggs the level of ecdysteroids was 41.05 ± 5.04 pg/mg fresh weight (±SEM, n=5). The level rapidly and significantly increased (P=0) to reach 121.66 ± 5.24 pg/mg fresh weight after 2 h and peaked at 4 h with a level of 166.58 ± 8.34 pg/mg fresh weight. Two hours later when the embryos underwent tracheation, the ecdysteroid levels began to decrease to 115.91 ± 7.07 pg/mg fresh weight and, then continuously decreased while tanning of the posterior spiracles and mouth hooks occurred. At 12 h after oviposition when hatching occurred, the ecdysteroid level had dropped back to 49.64 ± 4.32 pg/mg fresh weight. This level is not significantly different from that of newly laid eggs (Fig. 12).

In summary, the single peak of ecdysteroid titre midway during the embryonic development appears to initiate the development of embryonic tracheal trunks, followed by tanning of mouth hooks and spiracles.

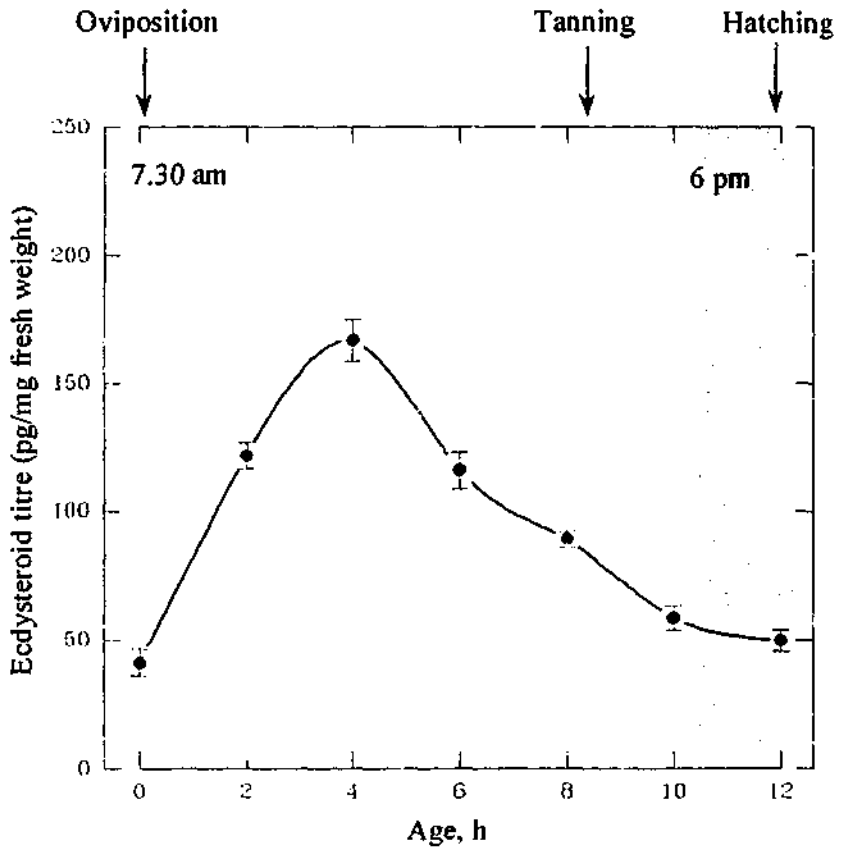


Fig. 12. Ecdysteroid titres in eggs measured by RIA throughout the embryonic stage of *Lucilia cuprina* reared at 27° C. Each point represents the mean value ± SEM of 5 replicates. Shaded area indicates the scotophase.

Table 2. A summary of developmental events ecdysteroid levels during the embryonic stages of *Lucilia cuprina* reared at 27° C.

Age		Ecdysteroid level	Developmental events	Reference for comparison in other species
h	min			
0	0	Low	**Oviposition**	
	5 - 15		Reduction division (two-cell stage)	
	15 - 30		Four-cell stage	
	30 - 45		Eight-cell stage	Fish (1947) a*
	30 - 45		Sixteen-cell stage	
	45 - 60		Thirty-two-cell stage	
2			Blastema, yolk cells and germ cells develop	
	215		Blastoderm clearly developed	Fish (1947) b*
4		Peak		
6 - 7			Segmental arrangement of the body	
8 - 9		Gradual decrease	Tracheal trunks apparent	
10 - 11			Posterior spiracles are sclerotised	
			Appearance of the gut	
			Mouth hooks are sclerotised	Wentworth (1982)#
			Embryos capable of movement	
12 - 13		Low	**Hatching**	

*Refers to *Lucilia sericata*
#Refers to *Neobellieria bullata*

3.2. Larval stages

In order to investigate the role of ecdysteroid hormones in regulating developmental events during larval stages, the insects were accurately staged and weighed, the moulting events analysed and the titre of ecdysteroids measured to test the hypothesis that an increase in ecdysteroid levels initiates each larval moult. Details of the statistical analysis are shown in Appendices 3 to 12.

3.2.1. Developmental aspect

3.2.1.1. Determination of larval instars

The larva of *L. cuprina* has a cephalopharyngeal apparatus consisting of a pair of mouth hooks, mandibular sclerites, paraclypeal phragma, and mandibular sclerites (Fig. 13). Examination of mouth hook development throughout larval stages revealed three distinct morphological groups corresponding to three instars as summarised in Table 3.

In the first instar, the mouth hook is shaped as a dorsally bent bar with a small hook near its anterior end and an average length of 26.06 ± 1.24 μm (± SEM, n=155) (Fig. 13). Newly hatched larvae (0 h) have mouth hooks with a length of 24.18 ± 0.98 μm (n=14) (Table 3). As the larvae grew, the size of mouth hooks increased progressively and reached a maximum of 28.32 ± 0.71 μm (n=20) at 10 h after hatching (P=0, Appendix 3). This was followed by a continuous decrease to 24.71 ± 0.78 μm (n=17) (P=0) up to 18 h. By that time the majority (92.8 %) of larvae had developed the new mouth hooks of the second instar and moulting was observed (Fig. 14). Thus, the age of 18 h can be taken as the end of the first instar and the start of the second instar. The whole process of

the first moult in the observed populations took place as early as 16 h and as late as 22 h after hatching.

Table 3. A summary of larval development of *Lucilia cuprina* as indicated by mouth-hook development.

Age (h)	Ecdysteroid level	mouth-hook length (mean ± SEM) (µm)	Developmental stage (%)*					Stage of development
			0	1	2	3	4	
0		24.18 ± 0.97	100	-	-	-	-	Hatching
2		24.92 ± 0.89	100	-	-	-	-	
4		25.44 ± 0.65	100	-	-	-	-	
6		26.02 ± 1.08	100	-	-	-	-	
8	First peak	28.09 ± 0.88	100	-	-	-	-	Instar I
10		28.32 ± 0.71	100	-	-	-	-	
12		26.61 ± 1.37	100	-	-	-	-	
14		25 90 ± 0.77	100	-	-	-	-	
16	Low	25.39 ± 0.90	95	5	-	-	-	
18		24.70 ± 0.78	7.1	35.7	57.1	-	-	Moult I
20		122.46 ± 10.98	5.6	5.6	38.9	22.2	27.8	
22		124.84 ± 4.07	-	-	-	-	100	
24		135.63 ± 2.96	100	-	-	-	-	
26	Second peak	135.26 ± 3.38	100	-	-	-	-	Instar II
28		118.75 ± 3.58	100	-	-	-	-	
30		116.56 ± 1.45	100	-	-	-	-	
32		116.28 ± 6.28	75	25	-	-	-	
34	Low	115.68 ± 1.44	27.2	72.8	-	-	-	Moult II
36		279.38 ± 16.57	6.5	3.2	19.3	61.3	9.7	
38		284.49 ± 16.81	-	-	-	12	88	
40		291.82 ± 9.08	-	-	-	-	100	
42		295.96 ± 7.97	100	-	-	-	-	
44		303.06 ± 14.86	100	-	-	-	-	
52		305.34 ± 9.95	100	-	-	-	-	Instar III
.								
.								
.								
128	Third peak							WPP

* 0, developing new mouth hook absent; 1, new hook present but not sclerotised; 2, new hook present with tip slightly sclerotised; 3, new hook present with 1/2 unsclerotised; 4, new hook 100 % sclerotised with the basal piece unsclerotised (Stages after Roberts, 1976); WPP, white-prepupal stage.

In the second instar, the mouth hooks were approximately five times larger than those of the first instar (Fig. 15) with an average length of 123.20 ± 7.68 µm (n=176). By 20 h following larval hatching, the mouth hook size was 122.46 ±

10.99 μm (n=16) and this apparatus had well developed components: *i.e.* the hook, the posterodorsal process, the anteroventral angle, and the basal piece (Fig. 15). The mediodorsal part of the mouth hook of the second instar is typically curved dorsally, as in the first instar. The maximum size was reached at 24 h with a length of 135.63 ± 2.96 μm (n=24) (P=0.000, Appendix 4). The size then decreased continuously to 115.68 ± 1.44 μm (n=20) at 34 h (P=0). At 32 h, 25% of the population had developed new third instar mouth hooks and by 34 h the number increased to 72.8% (Fig. 16). Thus, the second instar was considered to have ended by 34 h following hatching. The second larval moult took about 6 h to complete (32 - 40 h) in the observed populations.

In the third instar, the mouth hooks are approximately twice larger than those of the second stage with an average length of 293.35 ± 9.32 (n=189). In contrast with the second instar, the mediodorsal part of the mouth hook is ventrally curved and the proximal part of the hook is fused to the posterodorsal process (Fig. 17). In early third instar larvae (36 h) the average size of the mouth hook was 279.38 ± 16.58 (n=31). This then increased slowly but significantly (P=0.004, Appendix 5) to a size of 303.07 ± 14.87 μm (n=20) by 44 h. At 52 h, the size remained relatively constant (P=0.999) with a length of 305.35 ± 9.96 μm (n=20). No further mouth hook growth was detected until pupariation, which occurred at 128 h, marking the end of the third (or last) larval instar (Fig. 17).

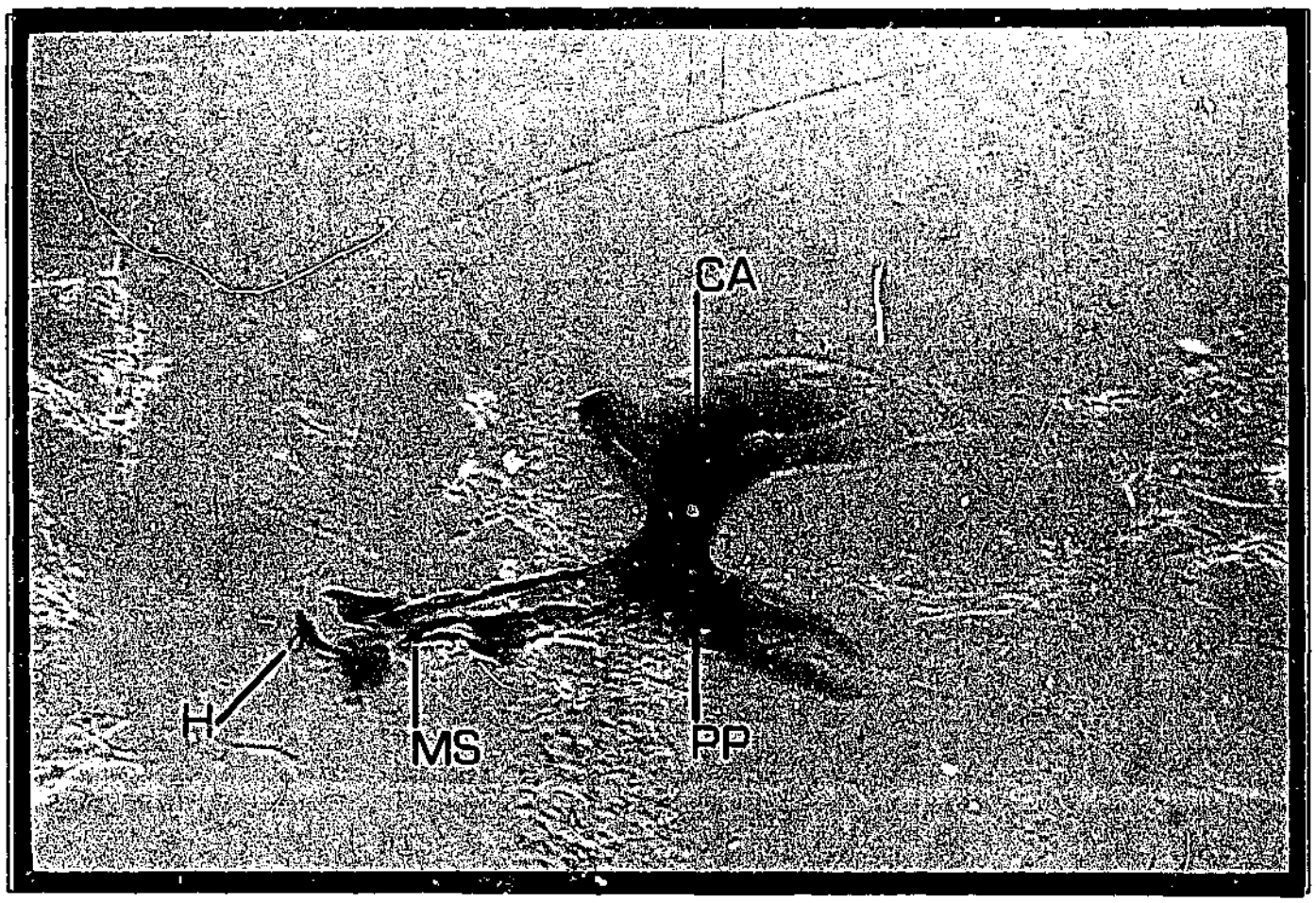
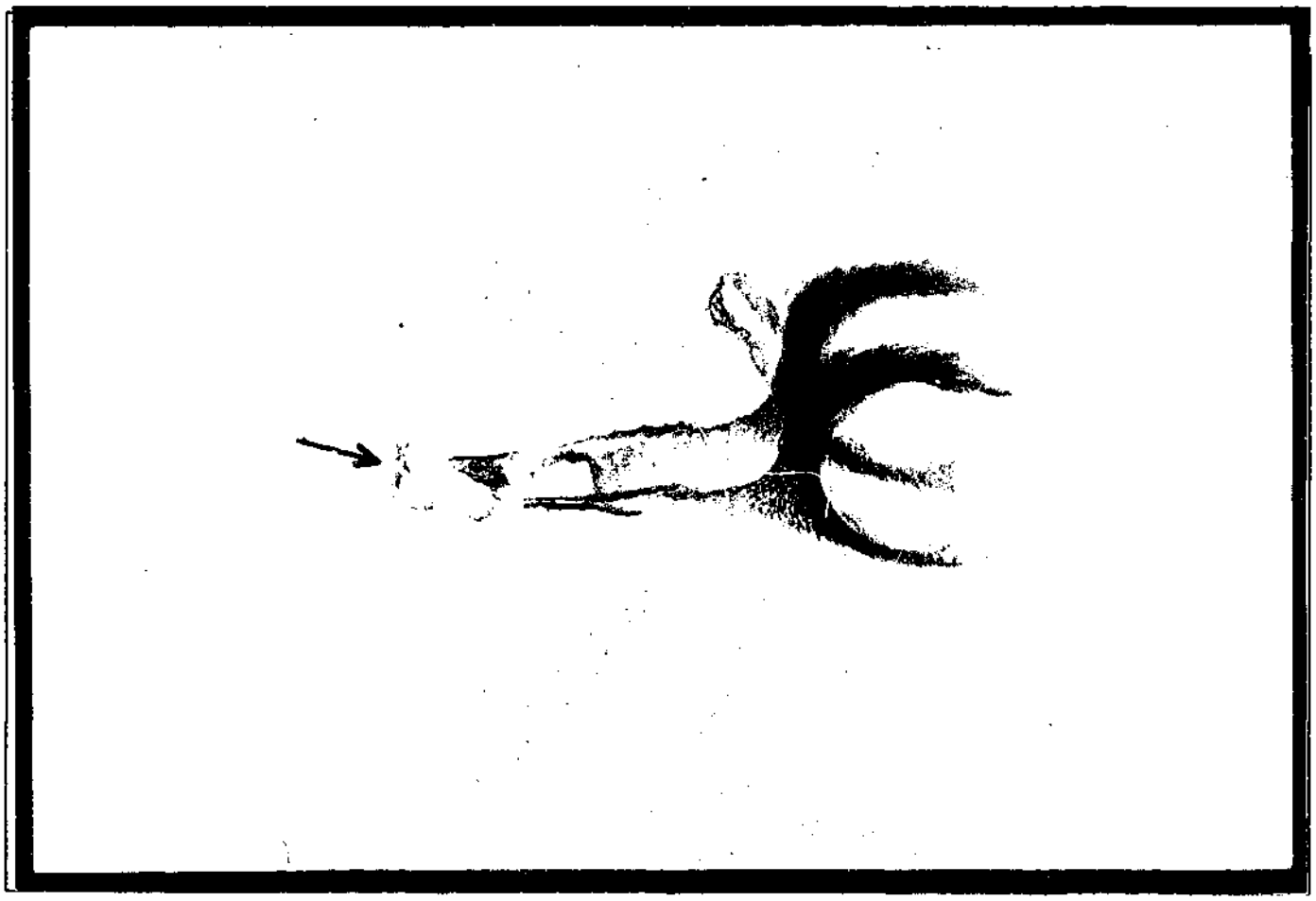


Fig. 13. Lateral view of the cuticular skeleton of the cephalopharynx of the first larval instar of *Lucilia cuprina*. CA, clypeal apodeme; H, mouth hook; MS, mandibular sclerite; PP, paraclypeal phragma. Nikon stereomicroscope with no filter.



100 μm

Fig. 14. Lateral view of the skeleton of the cephalopharynx during the first larval moulting of *Lucilia cuprina* at 18 h after hatching. The arrow indicates the second instar mouth hooks. Photographic conditions as for Fig. 13.

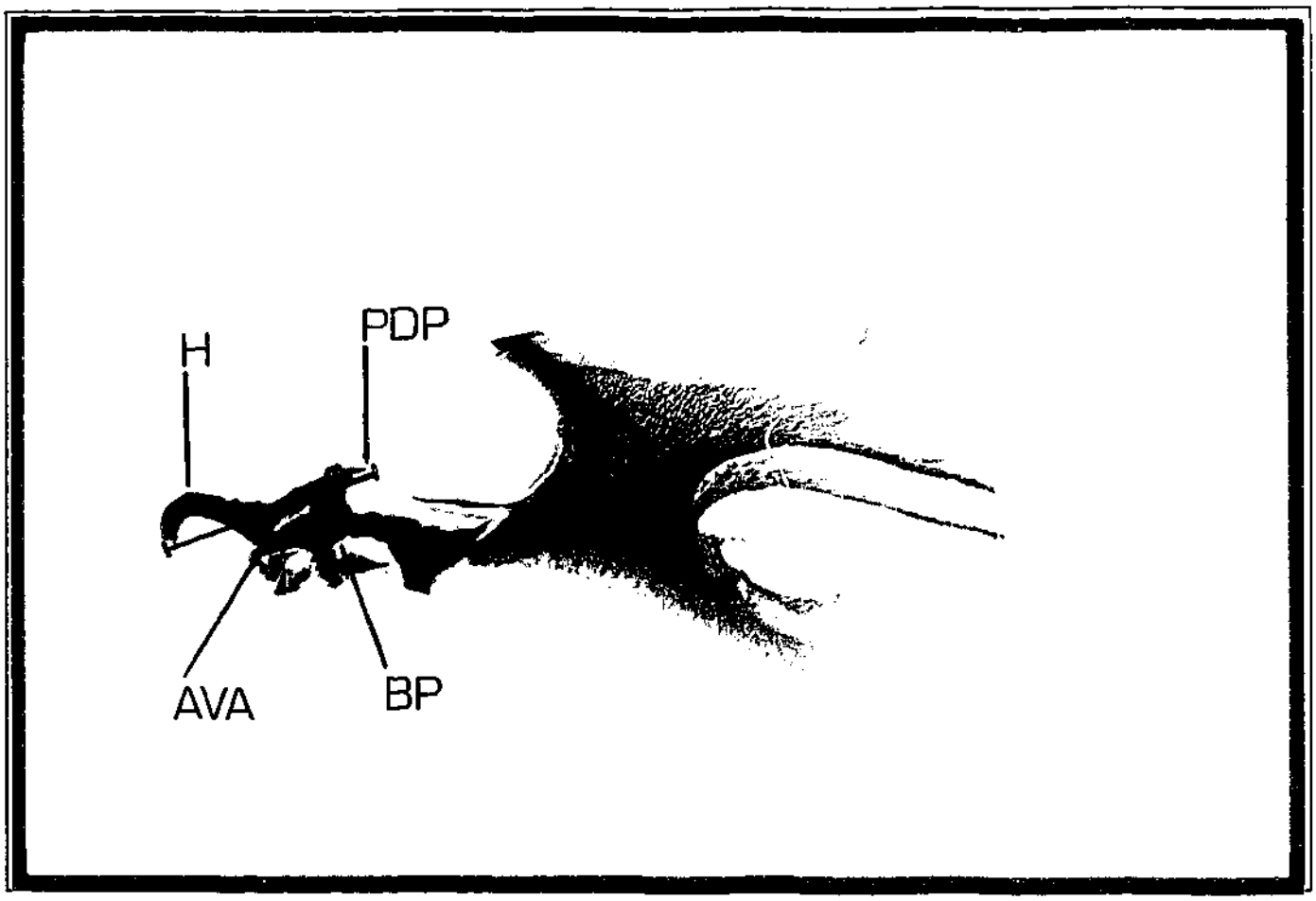
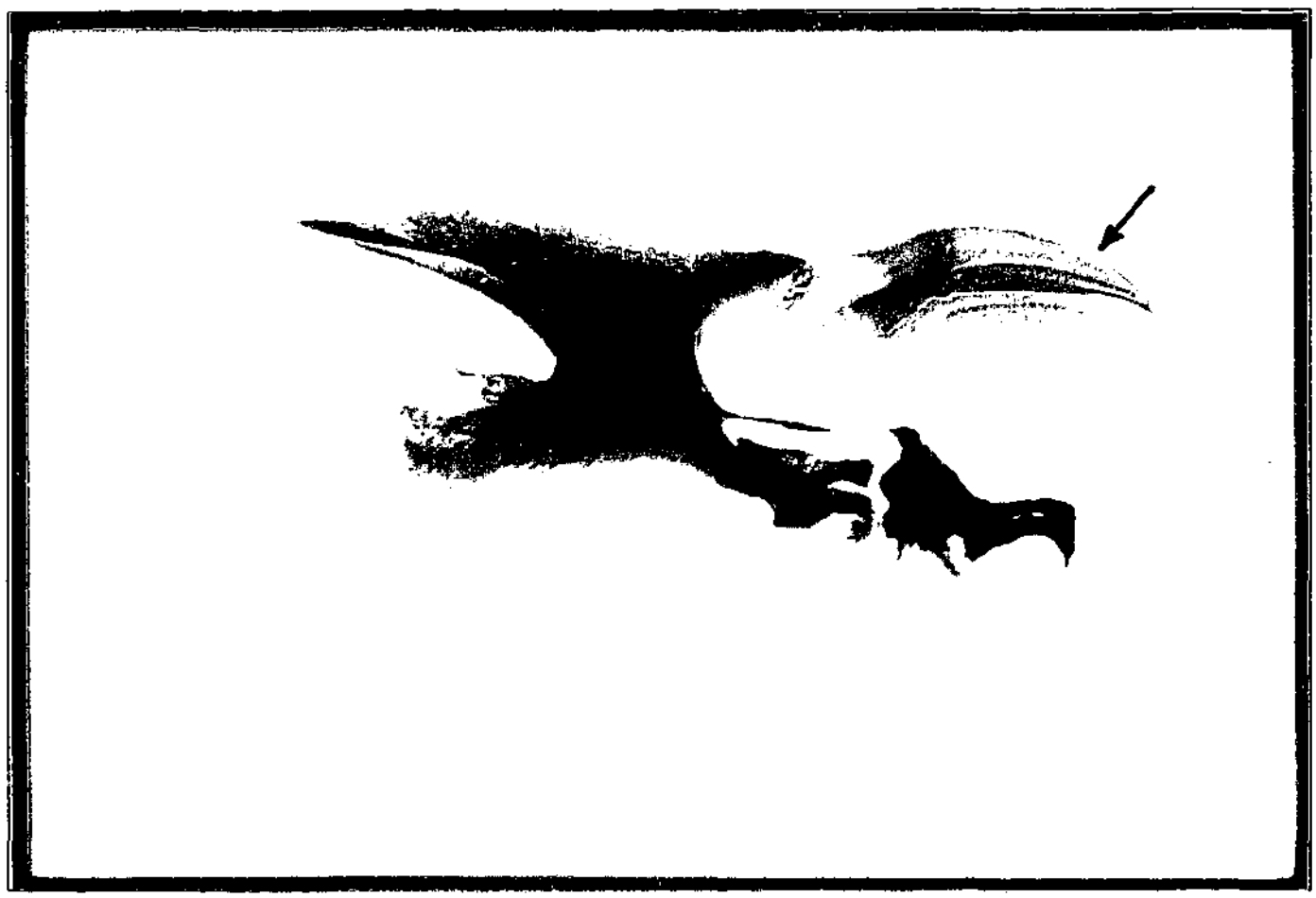


Fig. 15. Lateral view of the skeleton of the cephalopharynx of the second larval instar of *Lucilia cuprina*. H, mouth hook; PDP, posterodorsal process: AVA; anteroventral angle; BP, basal piece; Unlabeled line indicates the measurement points. Photographic conditions as for Fig. 13.



200 µm

Fig. 16. Lateral view of the skeleton of the cephalopharynx during the second larval moulting of *Lucilia cuprina* at 34 h after hatching. The arrow indicates the developing third instar mouth hooks. Photographic conditions as for Fig. 13.

400 µm

Fig. 17. Lateral view of the skeleton of the cephalopharynx of the third larval instar of *Lucilia cuprina*. Photographic conditions as for Fig. 13.

3.2.1.2. Formation of the PFL stage

About midway through the third instar, larvae left the decomposed liver and wandered in the breeding tray to seek a place for pupariation in the vermiculite under the liver boat. The pattern of the PFL stage formation under the breeding conditions was estimated by counting the larvae leaving the food during the last larval instar. Three groups of populations were examined: a first group developed from eggs laid between 0700 - 0800 h (Fig. 18, A), a second group was from eggs laid between 1430 - 1530 h (Fig. 18, B) and the third one was from eggs laid between 2330 - 2430 h (Fig. 18, C). In each group, several rearing medium replicates showed similar pattern of PFL formation (Fig. 18, A to C respectively). A mean curve for each group is shown in Fig. 19.

Examination of the first group of populations, developed from the eggs laid between 0700 - 0800 h, revealed that larvae started to leave the food by 95 h after hatching, or 0.5 h after the lights were off, with a low percentage of 0.08% entering the post-feeding stage (Figs. 18, A and 19). This percentage then increased slowly to reach 16.7% within 5 h and continued to increase rapidly thereafter. By the end of the scotophase (106 h after hatching) the number of PFL had reached 70% of the population. During the photophase, the rate of exodus was extremely low (0.3% average increase). In the next scotophase, the exodus from the liver resumed with more than 95% of the population having left the food source. The PFL formation rate was again very low during the following photophase. By the middle of the third scotophase (148 h) all third instar larvae had left the food.

The pattern of PFL stage formation from the second group of population, developed from eggs laid between 1430 - 1530 h, is shown in Figs. 18, B and 19. By 93 h after hatching (or 6.5 h after lights off) 0.3% of larvae had left the liver.

At the end of the first scotophase the number had increased to 24.7% at which time the age of larvae was 98 h. During the following photophase, the exodus slowed down and the total increase was only 2.2%. Exodus resumed early in the next scotophase (111 h) at which time 29.5% of the population had left the food, and the exodus rate increased rapidly and 82.5% of the population had enter the PFL stage by the end of the scotophase (123 h). The rate of the PFL formation then again decreased drastically in the next photophase. By 143 h or 3.5 h before the end of the last scotophase all larvae had left the food.

Examination of the third group of populations, developed from eggs laid between 2330 - 2430 h, revealed that larvae entered the PFL stage by 86 h after hatching near the end of the scotophase or 4.5 h before lights on (Figs. 18, C and 19). By the end of the first scotophase 6.7% of the larvae had left the food. The rate of exodus was remained low until approximately the middle of the next scotophase the number reached 25.6% of the population. By the end of the second scotophase 55.5% had entered the PFL stage. Again the rate of exodus almost ceased during the next photophase with the average increase of only 2.6%. By the end of the last scotophase (138 h) all the larvae had entered the PFL stage.

I conclusion, all three groups of populations showed that the formation of PFL of *L. cuprina* under the specific breeding conditions occurred during three consecutive nights, and the exodus from the food source was mostly confined to the scotophase. Very low rates of exodus occurred during the photophase. In the first group, the majority of the larvae entered the PFL stage by the end of the first exodus night, whereas in the second and third groups of larvae from eggs laid later in the day, the largest part of the exodus occurred during the second night (Fig. 19).

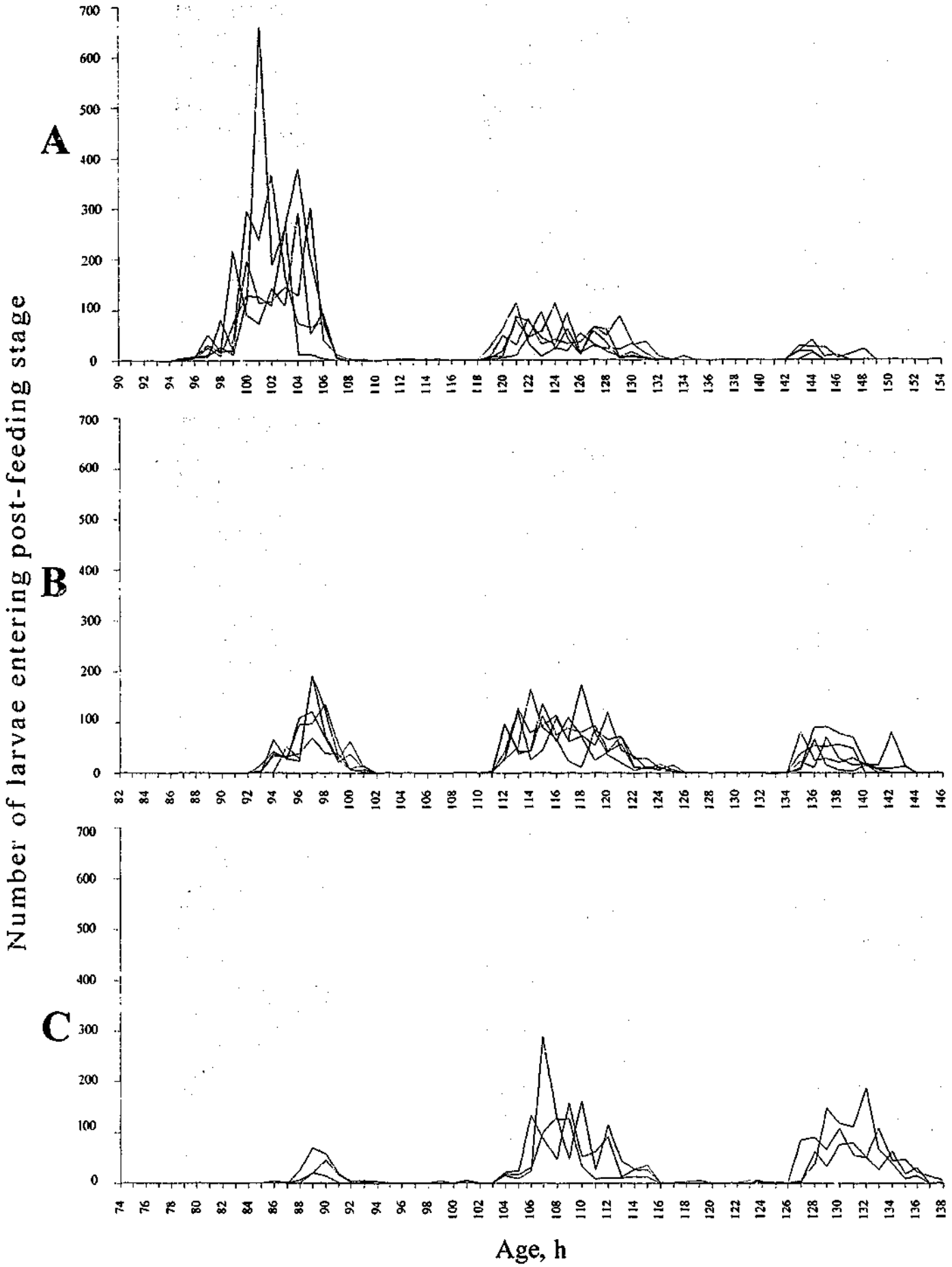


Fig. 18. Numbers of third instar larvae of *Lucilia cuprina* entering the PFL stage under 12 : 12, photo : scotophase, lights on at 0600 h local time. Three groups using eggs laid 0700 - 0800 h (A), eggs laid 1430 - 1530 h (B) and eggs laid 2330 - 2430 h (C) were compared. Shaded areas indicate the scotophase.

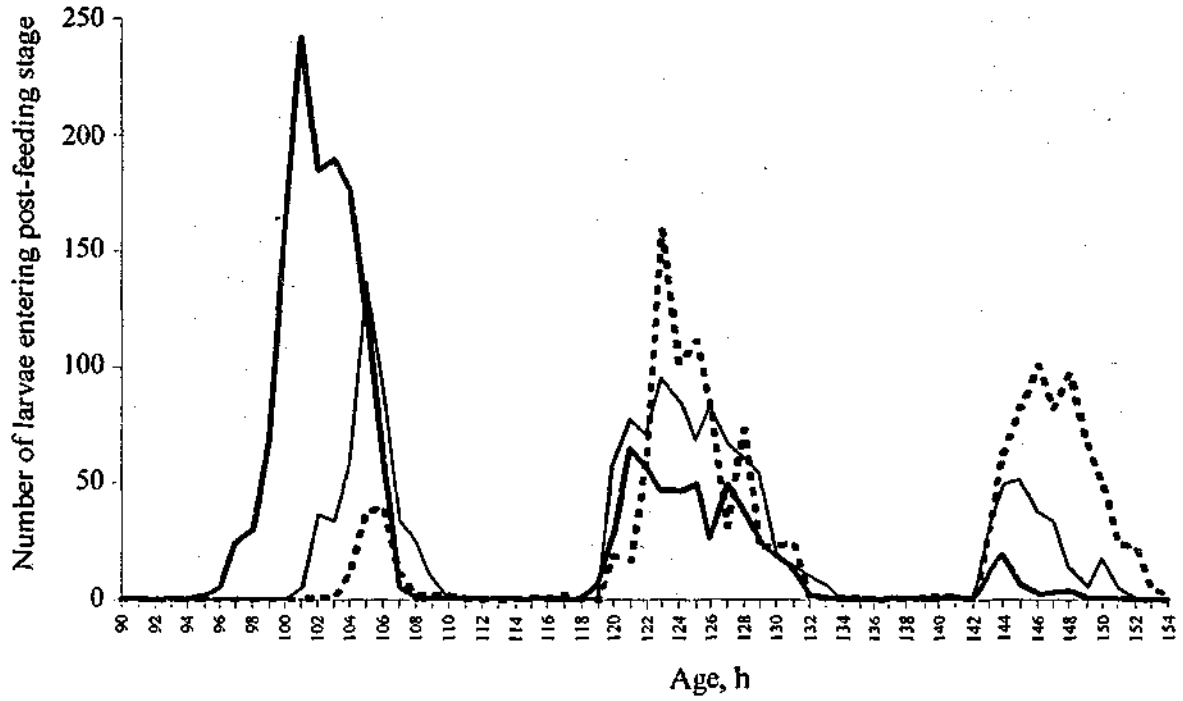


Fig. 19. Mean numbers of third instar larvae of *Lucilia cuprina* entering the PFL stage under 12 . 12, photo : scotophase lights on at 0600 h local time. Three groups eggs laid between 0700 - 0800 h (thick solid line), eggs laid between 14.30 - 15.30 h (thin solid line) and eggs laid between 23.30 - 24.30 h (dashed line) were compared. Shaded areas indicate the scotophase.

3.2.1.3. Body weight during larval stages

The mean weight of newly hatched larvae (0 h) was 0.064 ± 0.004 mg (± SEM, n=5) (Fig. 20). Larvae grew rapidly and by the first apolysis which occurred 18 h later, the weight of larvae had increased approximately eight fold to 0.53 ± 0.04 mg (n=5) (P=0, Appendix 6). During the second instar, rapid growth (P=0, Appendix 7) continued, and by the end of the second instar had increased fifteen fold. When the second moult occurred (34 h) the weight had reached 8.23 ± 0.09 (n=5). After entering the third instar, the weight doubled within 4 h (38 h after hatching) with a value of 17.52 ± 0.30 mg (n=5). The maximal weight of third instar larvae was reached by 80 h following hatching with a value of 52.81 ± 2.34 mg (n=5). Analysis of variance and regression throughout the feeding stage of third instar (34 - 96 h) showed a significant increase (P=0, Appendix 8); while during post-feeding stage, the weight of wandering larvae decreased significantly (P=0.019, Appendix 8) to reach a value of 44.17 ± 2.18 mg (n=10) by the time pupariation started at 128 h.

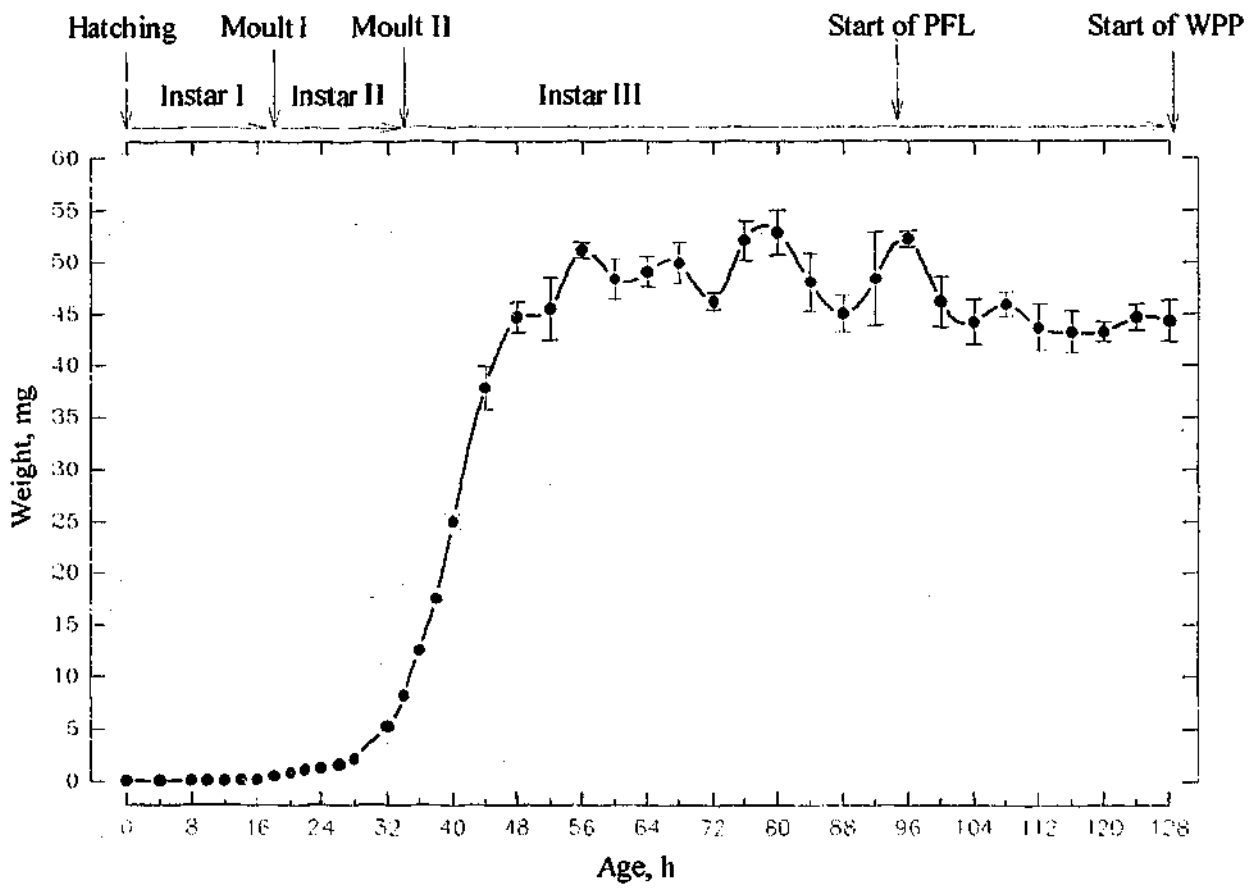


Fig. 20. Mean weight of larval *Lucilia cuprina* shown in mg/individual. Each point represents the mean weight ± SEM of 5 - 10 replicates, except where too small to show. PFL = post-feeding larval stage, WPP = white prepupal stage. Shaded areas indicate the scotophase.

3.2.2. Endocrinological aspect

3.2.2.1. Ecdysteroid titres during the first larval instar

The RIA measurement of whole-body ecdysteroids during the first larval instar of *L. cuprina* is shown in Fig. 21. The details of the statistical analysis are shown in Appendix 9.

The mean level of ecdysteroids in the newly hatched larvae (0h) was relatively high at 49.63 ± 4.32 pg of 20-hydroxyecdysone equivalent/mg fresh weight (Fig. 21). Within 8 h following hatching, the titre increased to a maximum of 99.49 ± 8.61 pg/mg fresh weight. This single significant peak (P=0) occurred during the scotophase, some 10 h prior to the first larval moult. The titre then cominuously decreased until the end of the first instar to a low value of 19.71 ± 0.68 pg/mg fresh weight (P=0.007).

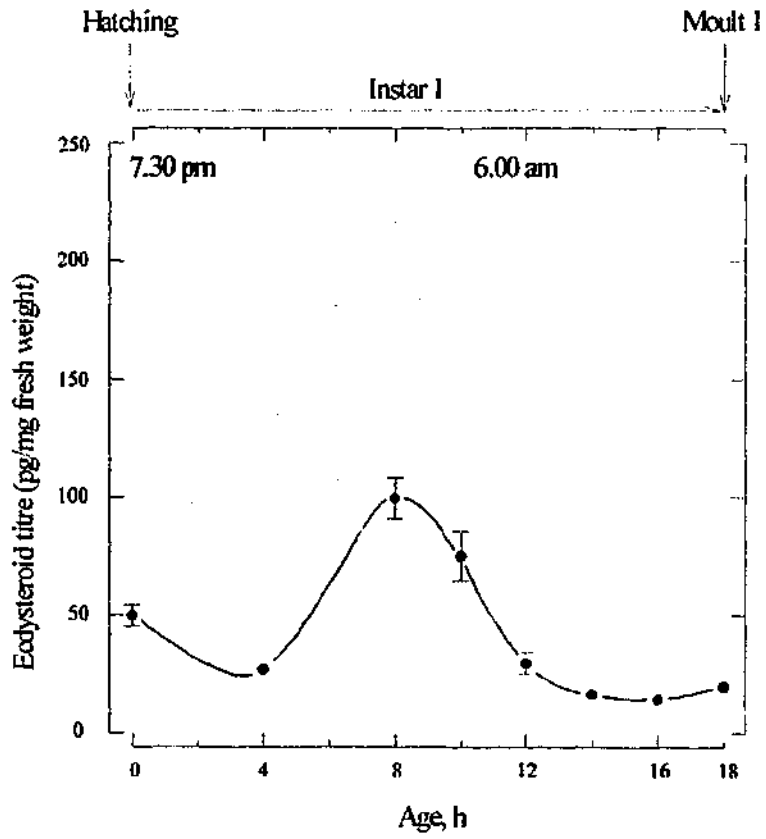


Fig. 21. Ecdysteroid titres from whole-body homogenates measured by RIA in the first instar larvae of *Lucilia cuprina* reared at 27° C. Each point represents the mean value ± SEM (except when too small to show) of 5 - 10 whole-body replicates. Shaded area indicates the scotophase.

3.2.2.2. Ecdysteroid titres during the second larval instar

The RIA quantification of ecdysteroids from whole-body extracts of the second larval instar is shown in Fig. 22. Statistical analysis details are shown in Appendix 10.

After undergoing the first larval moult, *i.e.* 18 h following hatching, no significant changes (P=0.094) occurred in ecdysteroid titre (19.71 - 21.88 pg/mg fresh weight) for at least the next four hours (Fig. 22). Shortly after the next scotophase had begun, the level increased rapidly and reached a maximum value of 61.98 ± 2.40 pg/mg fresh weight at 26 h. A sharp decrease in ecdysteroid level followed: within 2 h the titre had dropped to a low value of 11.69 ± 1.39 pg/mg fresh weight, and remained low until the second larval moult. This significant ecdysteroid peak (P=0) occurred some 8 h before the second larval moult occurring 34 h after hatching.

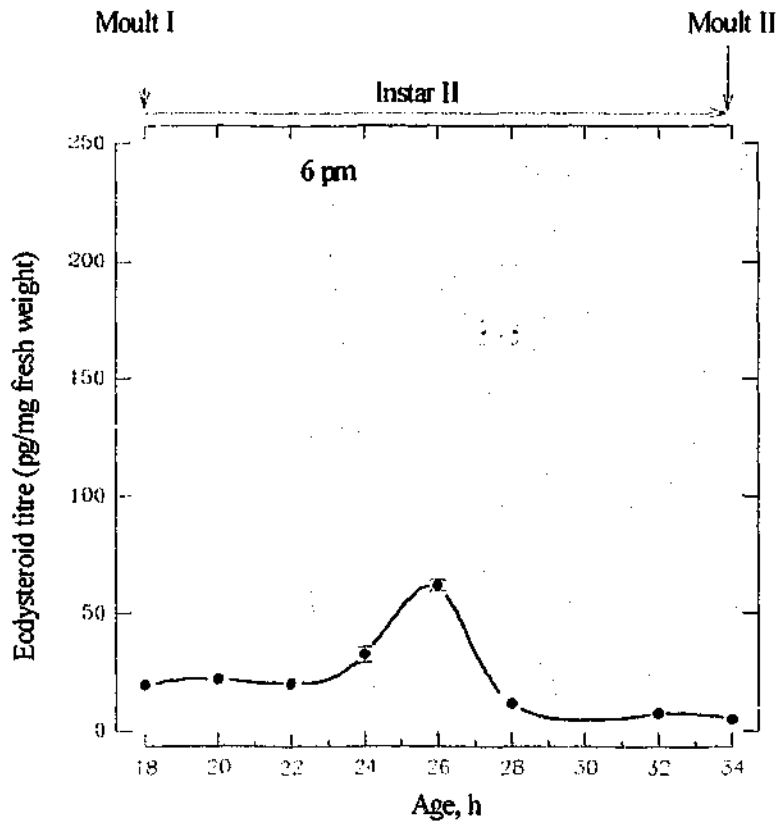


Fig. 22. Ecdysteroid titres from whole-body homogenates measured by radioimmunoassay in the second instar larvae of *Lucilia cuprina* reared at 27° C. Each point represents the mean value ± SEM (except when too small to be shown) of 5 -10 whole-body replicates. Shaded area indicates the scotophase.

3.2.2.3. Ecdysteroid titres during the third larval instar

The ecdysteroid titres during the third larval instar is shown in Fig. 23. The details of statistical analysis are shown in Appendices 11 and 12.

After the second larval moult (34 h), the ecdysteroid level in the early third instar larvae remained constant and relatively low (4.81 - 10.86 pg/mg fresh weight) (P=1 to 0.97). The titre then remained relatively constant (Appendix 11) until the end of the feeding stage (96 h) (Fig. 23).

After entering the PFL stage, the ecdysteroid titre remained relatively constant (Appendix 12) at least until 116 h following hatching. A peak occurred at 128 h, reaching a maximum of 240.37 $\pm$ 23.91 pg/mg fresh weight (P=0, Appendix 12) during the last scotophase. This peak coincided with the start of pupariation.

Fig. 24 summarises the ecdysteroid profile throughout the embryonic and larval stages. One peak of ecdysteroid titres occurred midway of the embryonic development, followed by tracheation and tanning of the posterior spiracles. A first larval ecdysteroid peak occurred during scotophase 8 h after hatching and initiated the first instar moult. A second larval ecdysteroid peak occurring during scotophase at 26 h after hatching initiates the second instar moult. At the end of the PFL stage, a great ecdysteroid peak occurred during the scotophase and was followed by pupariation.

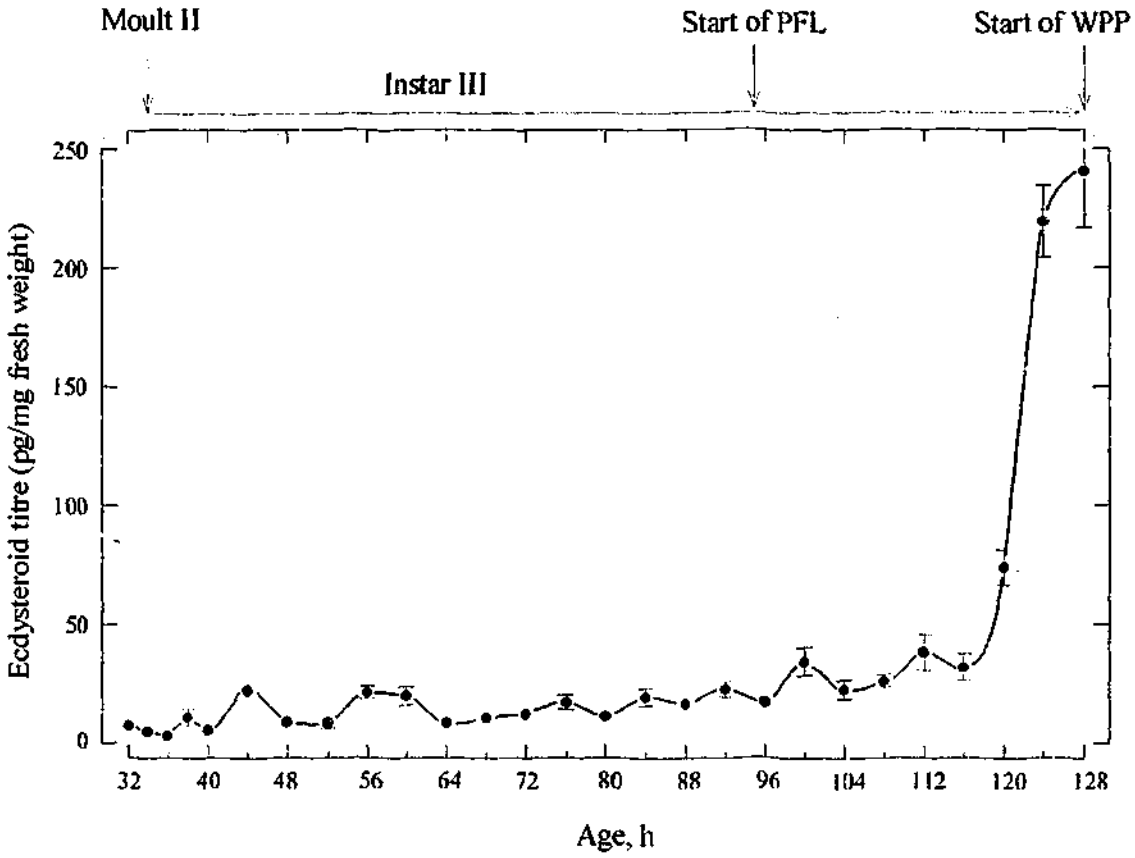


Fig. 23. Ecdysteroid titres from whole-body homogenates measured by RIA in the third instar larvae of *Lucilia cuprina* reared at 27° C. Each point represents the mean value ± SEM of 5 - 10 whole-body replicates, except where too small to show. Shaded areas indicate the scotophase.

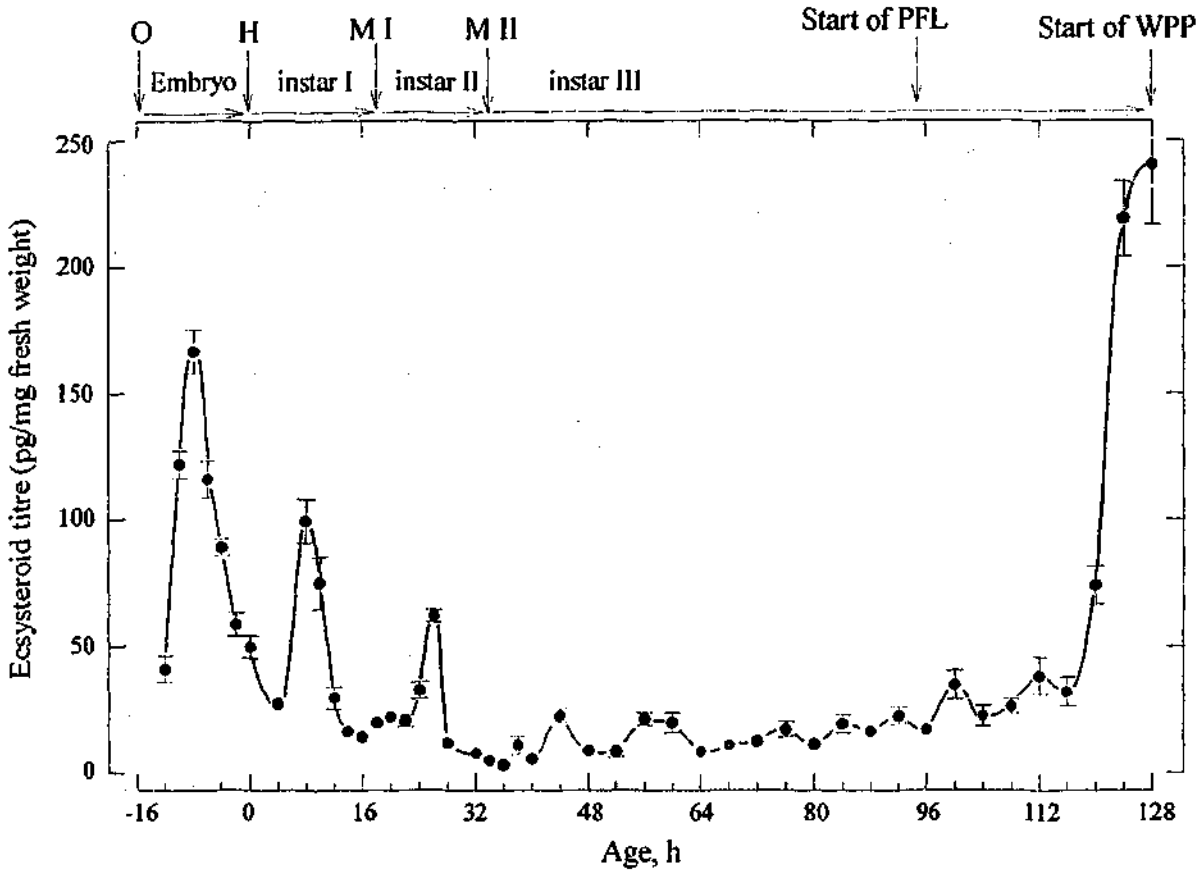


Fig. 24. A summary of the ecdysteroid titres measured by RIA during embryonic and larval stages of *Lucilia cuprina* reared at 27° C. Each point represents the mean value ± SEM of 5 whole-body replicates, except where too small to show. Newly hatched larvae were designated as 0 h of age; O, ovipositition; H, hatching; MI, moult I; MII, moult II; PFL, post-feeding larval stage; WPP, white prepupal stage. Shaded areas indicate the scotophase.

3.3. Prepupal, pupal and pharate adult stages

In order to study the role of ecdysteroids in regulating developmental processes during metamorphosis, developmental events occurring in prepupal, pupal and pharate adult *L. cuprina* were observed and ecdysteroid titres measured at four hour interval as previously described.

3.3.1. Developmental aspects

3.3.1.1. Prepupal stage

At the end the post-feeding stage (128 h after hatching), the larva began to contract gradually and became rounded in shape. These events mark the start of pupariation which leads to the formation of the prepupal stage. At this time the larval cuticle was still white and flexible, but started to tan within one hour and continued to darken and harden to form the puparium.

Microscopic examination of sagittal sections of white prepupae revealed that, at this stage, the epidermis is still firmly attached to the puparium over the entire body (Fig. 25, A to C). The epidermal cell nuclei were elongated and the larval muscles underneath the epidermis were still striated (Fig. 25, B). As shown in Fig. 25, C, part of the muscle had lost its striation indicating that muscle degeneration had begun. By 8 h after pupariation, the epidermis at the anterior part of the body had separated from the puparium marking the end of the prepupal stage (Fig. 26, A). This is clearly demonstrated in Fig. 26, B.

3.3.1.2. Pupal stage

Histological examination during the pupal stage showed that the separation of the epidermis from the puparium started 8 h after pupariation, marking the beginning of the pupal stage (Fig. 26, A). This apolysis and subsequent cuticle secretion started from the anterior part of the body (Fig. 26, A). During this period the epidermal cells were approximately one and a half times thicker than in the prepupal stage and their nuclei rounded. Moreover, the striated muscles had commenced autocytolysis resulting in the breakdown of the larval tissues (Fig. 26, B).

Four hours later (12 h after pupariation) the entire epidermis was completely separated from the puparium, marking the beginning of the cryptocephalic pupal stage. At this stage the shape of the pupa still very much resembled that of a larva, since a thorax and an abdomen could not be distinguished (Fig. 27, A). The deposition of the pupal cuticle continued and the space between the pupal cuticle and the puparial wall was filled with a fluid, presumably the moulting fluid (Fig. 27, B).

Twenty-four hours after pupariation many changes had taken place, and thorax and abdominal regions could easily be distinguished in the pupal body. At this stage the abdominal part showed segmentation and occupied about three quarters of the whole body. A large gas bubble filled the gut occupying the anterior part of the abdomen (Fig. 28).

At 28 h following pupariation, the phanerocephalic pupa was being formed. The head of the presumptive adult was being evaginated from the thorax as shown in Fig. 29. Furthermore, the gas bubble had disappeared at this stage.

The abdomen was deflated and may have contracted to evaginate the head and thorax appendages.

By 40 h the pupal/adult apolysis had taken place. The epidermis had completely separated from the pupal cuticle and the pharate adult had formed (Fig. 30). At this stage the body shape resembled that of the adult.

Fig. 25, A A sagittal section of a white prepupa of *Lucilia cuprina*. The area indicated by the top arrow is magnified in Fig. 25, B and by the bottom arrow in Fig. 25, C. Nikon stereomicroscope with green back filter, Toluidine blue stain.

Fig. 25, B. A magnified view of the dorsal area of the white prepupa indicated by the top arrow in Fig. 25, A. Note the epidermis (EP) still attached to the puparium (P). Underneath the epidermis lies larval striated muscle. Leitz Orthoplan microscope, green 520 mm filter, Toluidine Blue stain.

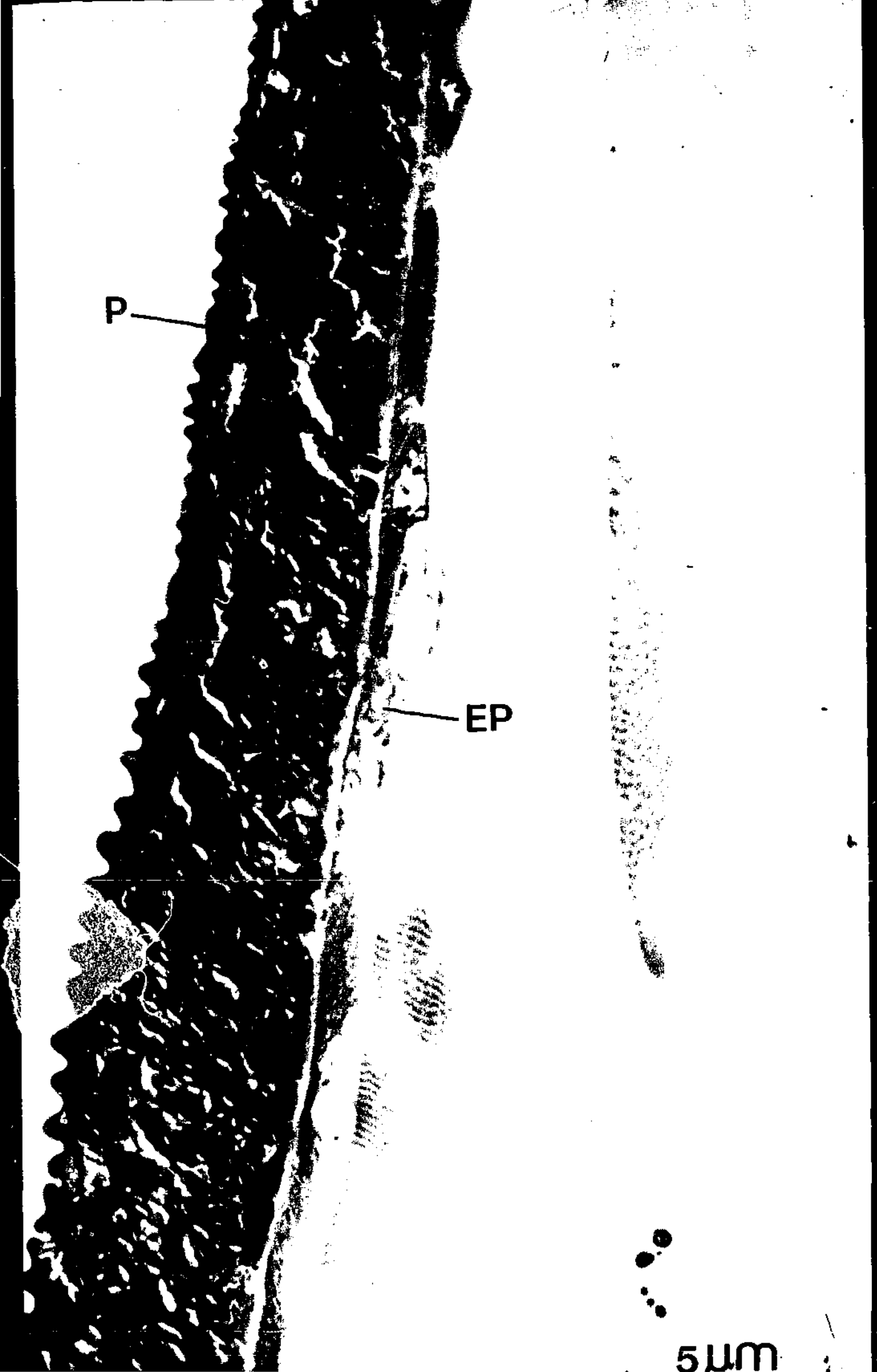

P
EP
5µm

Fig. 25, C. Magnified view of the ventral area of the white prepupa (bottom arrow in Fig. 25, A). Note the epidermis (EP) still attached to the puparium (P). The muscle underneath the epidermis has lost its striation. Leitz Orthoplan microscope, green 520 mm filter, Toluidine Blue stain.

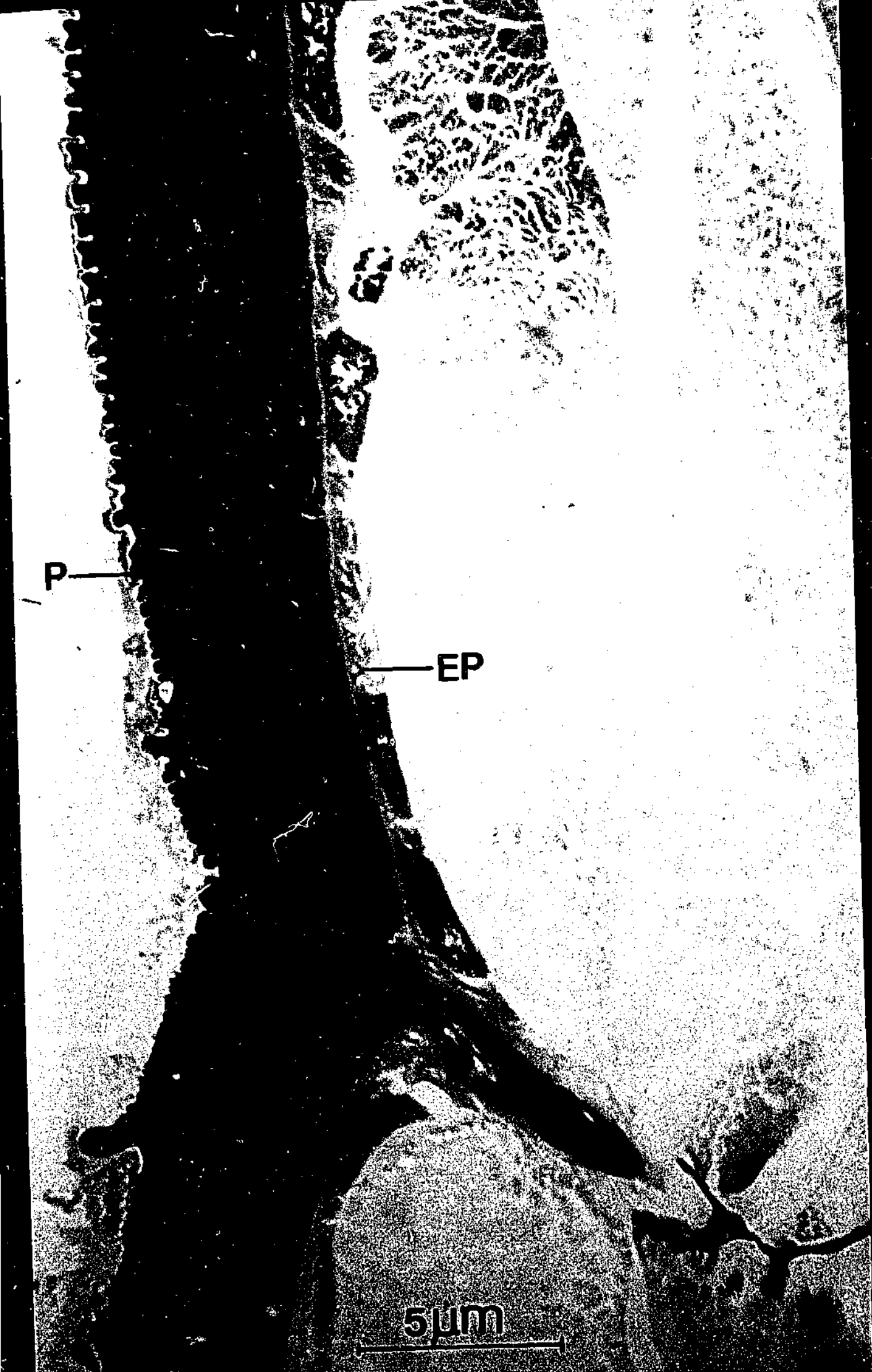

P
EP
5µm

Fig. 26. A. A sagittal section of *Lucilia cuprina* during larval/pupal apolysis at 8 h after pupariation. Note the anterior part (right) of the epidermis has separated from the puparium. EP, epidermis; P, puparium. The arrow indicates the magnified area shown in Fig. 26, B. Nikon stereomicroscope with green back filter, Toluidine blue stain.

Fig. 26, B. Magnified view of the integument during larval/pupal apolysis at the area indicated by the arrow in Fig. 26, A. Note the epidermis (EP) has been partly separated from the puparium (P) and the pupal cuticle (PC) is being deposited. The muscle underneath the epidermis has lost its striation. N, epidermal cell nucleus. Leitz Orthoplan microscope, green 520 mm filter, Toluidine Blue stain.

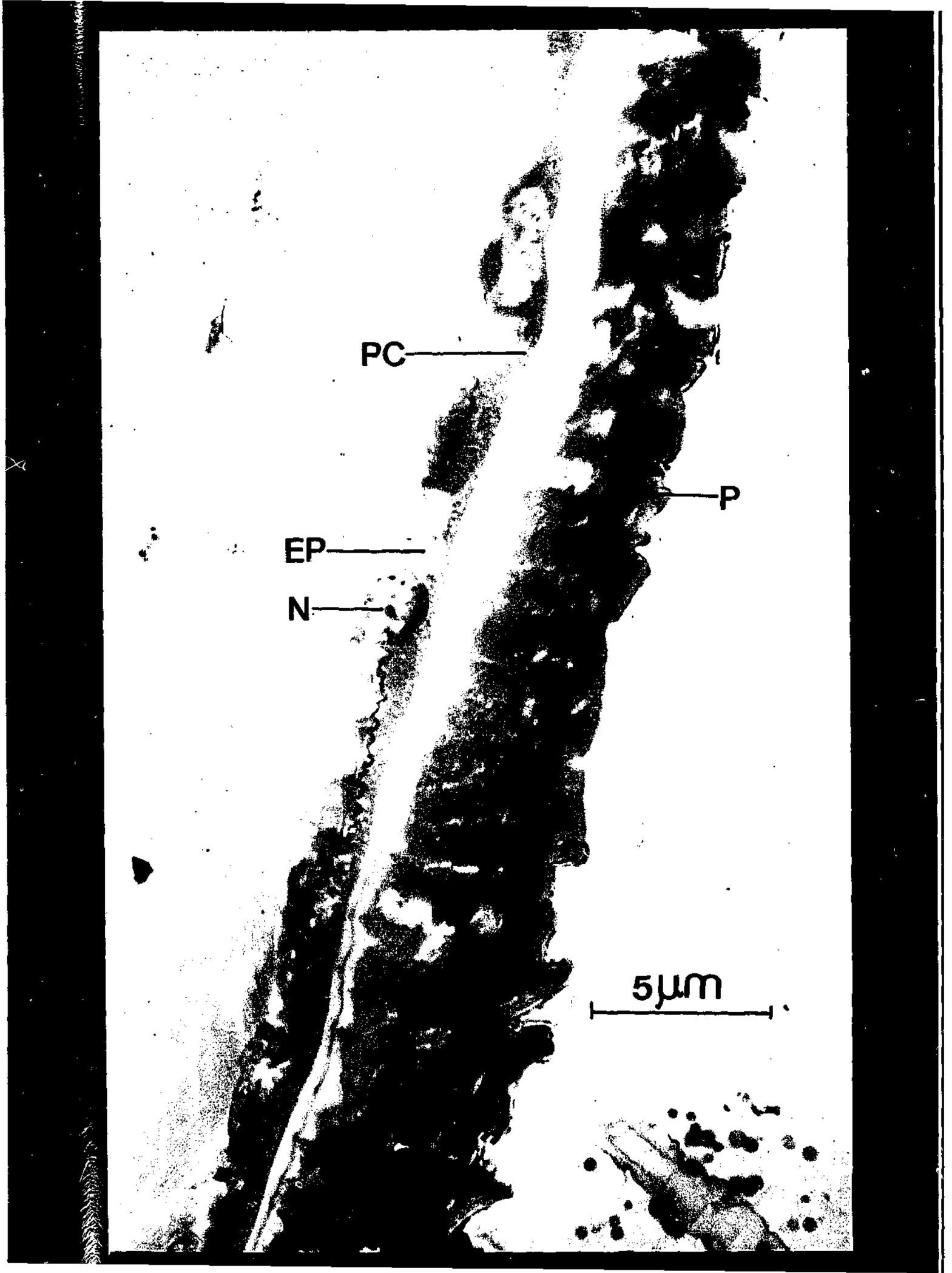

PC
P
EP
N
5µm

Fig. 27, A. A sagittal section of a pupa of *Lucilia cuprina* at 12 h after pupariation. Note the epidermis has separated from the puparium all over the body. EP, epidermis; P, puparium. The area indicated by the arrow is magnified in Fig. 27. B. Nikon stereomicroscope with green back filter, Toluidine blue stain.

Fig. 27, B. Magnified view of a sagittal section of 12 h pupa at the area indicated by the arrow in Fig. 27, A. PC, pupal cuticle; P, puparium; MF, moulting fluid. Leitz Orthoplan microscope, green 520 mm filter, Toluidine Blue stain.


PC
MF
P
5µm

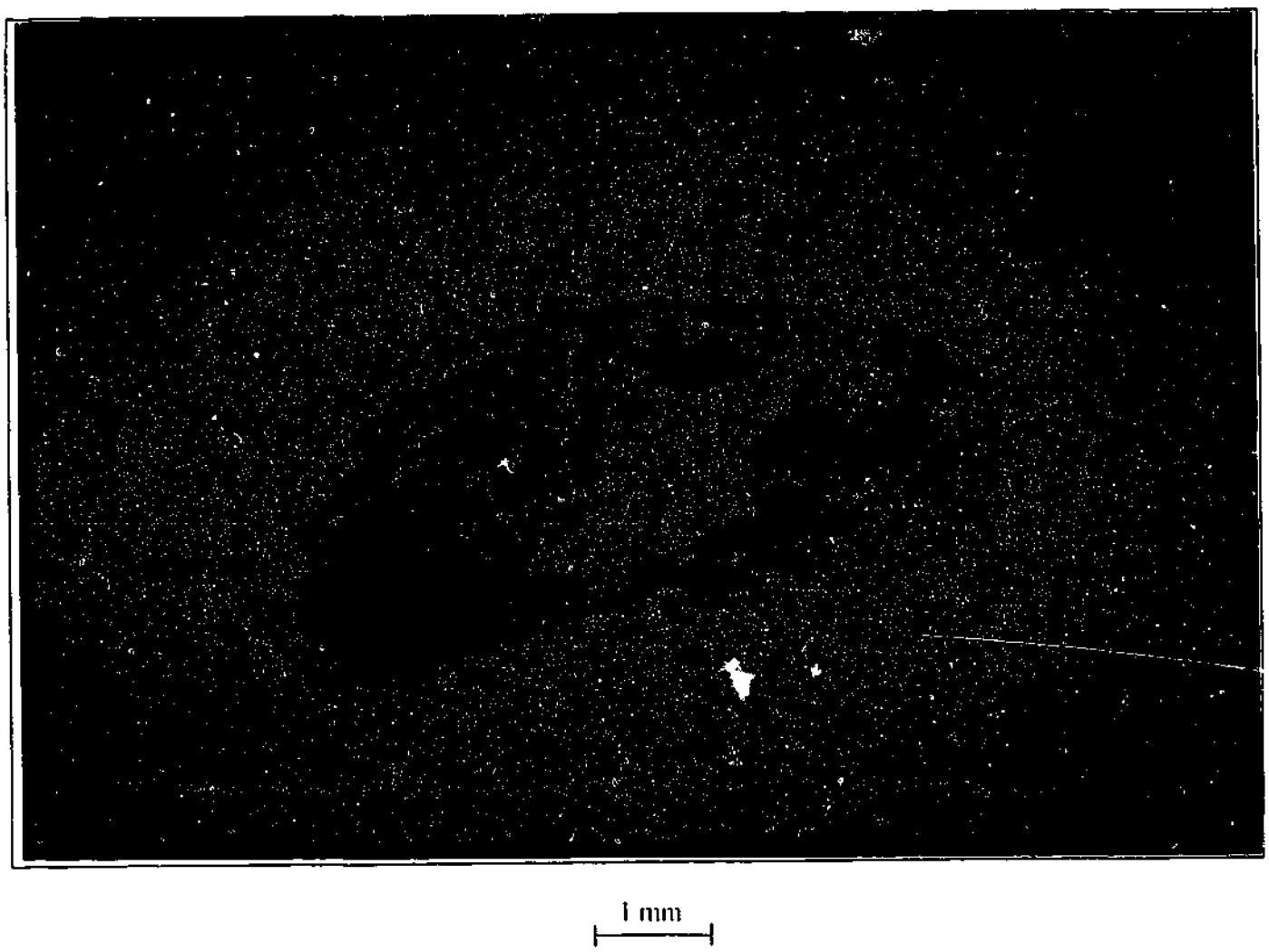


Fig. 28. A sagittal section of a cryptocephalic pupa of *Lucilia cuprina* at 24 h after pupariation. P, puparium; PC, pupal cuticle; TH, thorax; AB, abdomen; B, gas bubble. Nikon stereomicroscope with green back filter, Toluidine blue stain.

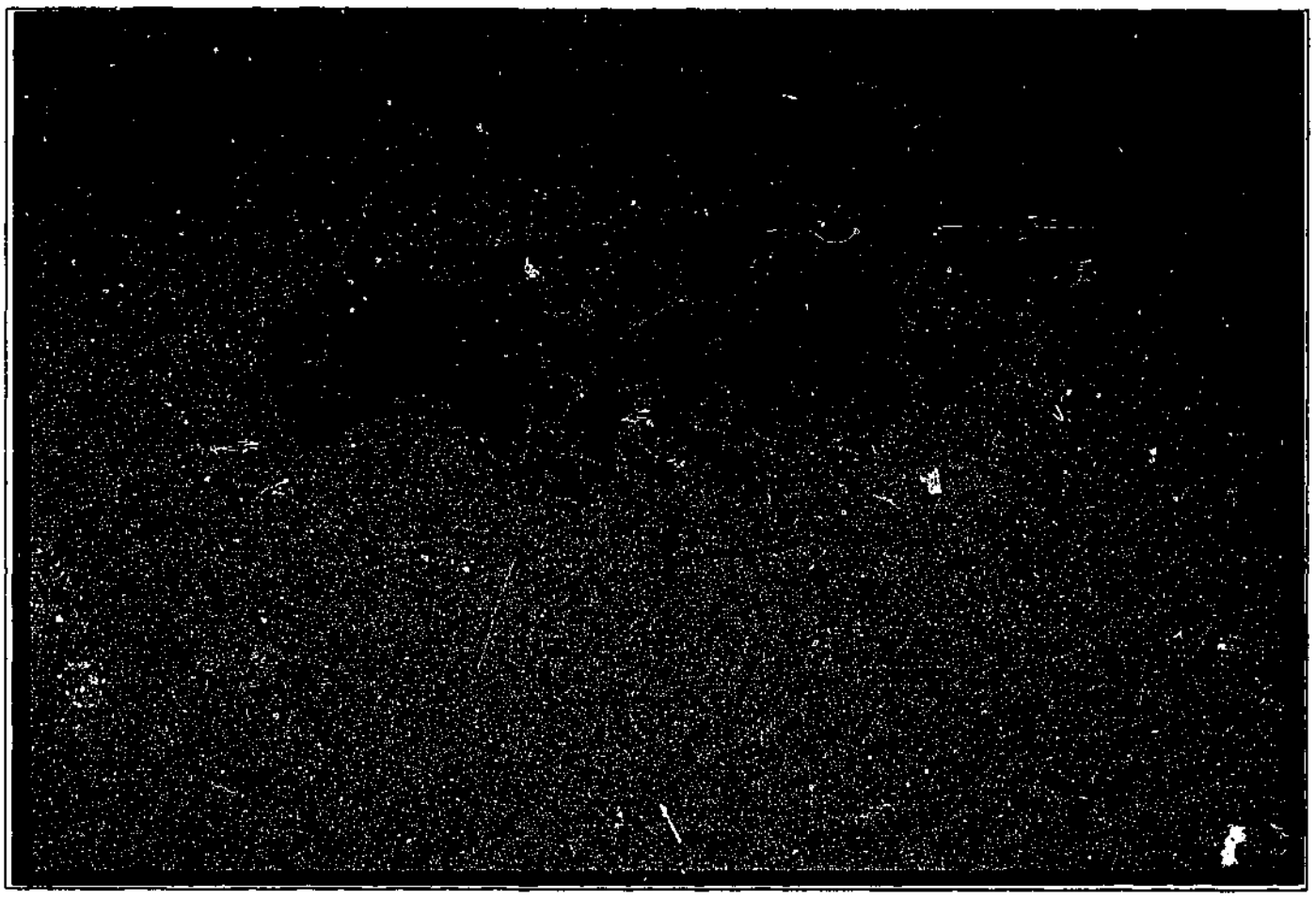


Fig. 29. A sagittal section of a pupa of *Lucilia cuprina* during the evagination of the head to form a phanerocephalic pupa at 28 h after pupariation. Note that the head is being evaginated and the brain is migrating into the head and the air bubble has disappeared from the gut. P, puparium; PC, pupal cuticle; B, brain; C, head; TH, thorax; AB, abdomen. Nikon stereomicroscope with green back filter, Toluidine blue stain.

Fig. 30. A sagittal section of a pharate adult of *Lucilia cuprina* at 40 h after pupariation. Note the epidermis has been separated from the pupal cuticle all over the body. P, puparium; EP, epidermis; PC, pupal cuticle; B, brain. Nikon stereomicroscope with green back filter. Toluidine blue stain.

3.3.1.3. Body weight during prepupal, pupal and pharate adult stages

3.3.1.3.1. White prepupae (WPP)

The average weight of female *L. cuprina* at the formation of WPP (0 h) was 46.17 ± 0.22 mg (±SEM, n=188) whereas that of the male was 36.34 ± 0.22 (n=130). The maximum weights were 56.14 mg for the females and 40.78 mg for the males, while the minimum weights were 39.02 mg and 27.65 mg respectively. Statistical analysis using t-Test revealed that the females were significantly heavier than the males (P=0, Appendix 13). The frequency distribution of white-prepupal weight of both sexes of *L. cuprina* is shown in the histogram in Fig. 31. The overlapping weight area lies between 39.02 and 40.78 mg. Thus, sexual dimorphism in weight can be used for sexing prepupae by discarding the animals whose weights fall in or near this range.

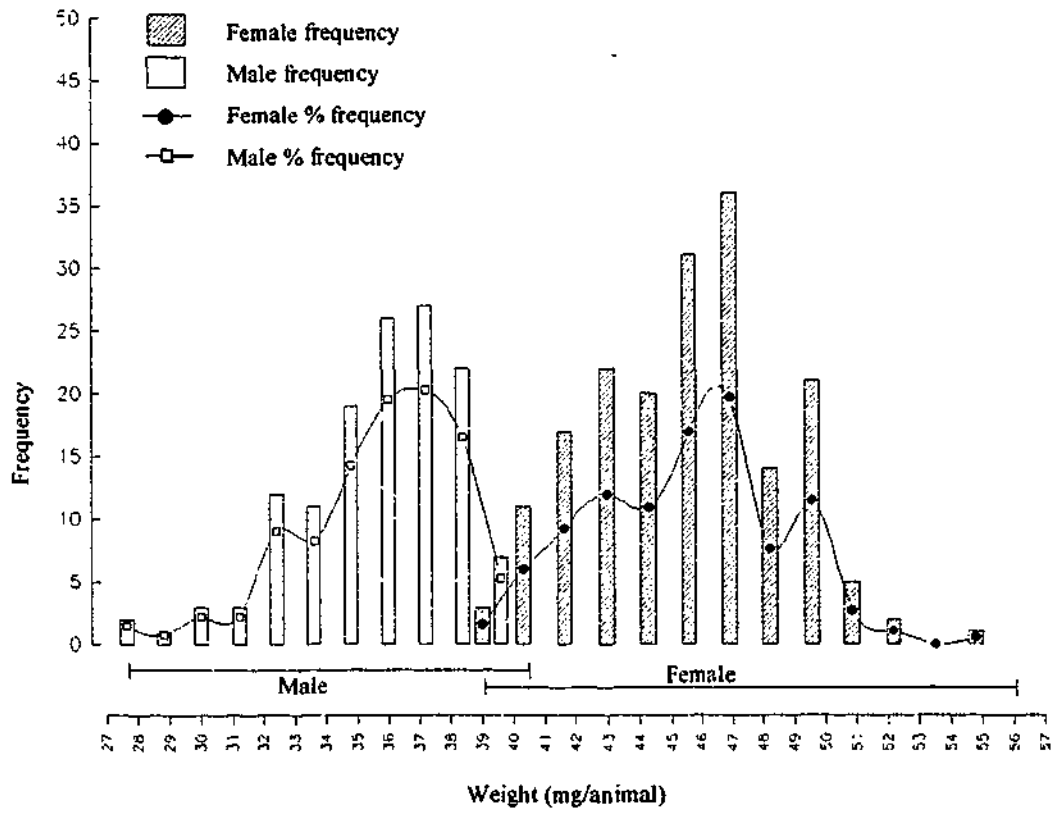


Fig. 31. A histogram of frequency distribution of white-prepupal weight of both sexes of *Lucilia cuprina*, reared at 27° C under 12 : 12, photo-scotophase. Each vertical bar represents the frequency of the weight range from the represented point to the next point, represented by the next bar, except the last bars whose next point is the maximum weight, indicated by the horizontal lines.

3.3.1.3.2. Prepupae, pupae and pharate adults

The mean body weight (±SEM, n=5) during the development of prepupae, pupae and pharate adults of both sexes is shown in Fig. 32. Regression analysis from 0 - 8 h after pupariation revealed that the mean body weight of female prepupae differed significantly between ages (P=0.0034), but for the males this was not the case (P=0.2824) (Appendix 14). During this 8 h period, the mean weight decreased from 50.40 ± 1.24 mg to 44.90 ± 0.37 mg for females and from 36.14 ± 0.62 mg to 33.51 ± 1.09 for males.

Analysis of regression after the animal underwent larval/pupal apolysis (8 h) following pupariation and entered the pupal stage until the next apolysis (36 h) revealed that the female body weights continued to decrease significantly to 43.03 ± 0.61 (P=0.0199). In males, however, there was no significant variation in weight between the ages (P=0.406). The male average weight at 36 h was 33.46 ± 0.35 mg (Appendix 15) and remained constant throughout the pupal stage.

Regression analysis during the pharate adult stage which started from pupal/adult apolysis (36 h) until eclosion, showed significant decrease in weights in both sexes (P=0, Appendix 16). When the adult flies started to emerge at 160 h, the mean weight was 39.33 ± 0.62 mg for females and 30.42 ± 0.73 mg for males. Briefly, the weight of males started decreasing significantly only after the pharate-adult apolysis, while that of females started decreasing earlier, *i.e.* almost as soon as pupariation had occurred.

Finally, analysis of variance and regression for body weights throughout prepupal, pupal and pharate-adult stages (0 - 160 h) revealed that both female and male body weights decreased significantly over these developmental stages (P=0,

Appendix 17). Comparison between female and male weights using paired-sample t-Test throughout prepupal, pupal and pharate-adult stages revealed that females were significantly heavier than males (P=0, Appendix 18).

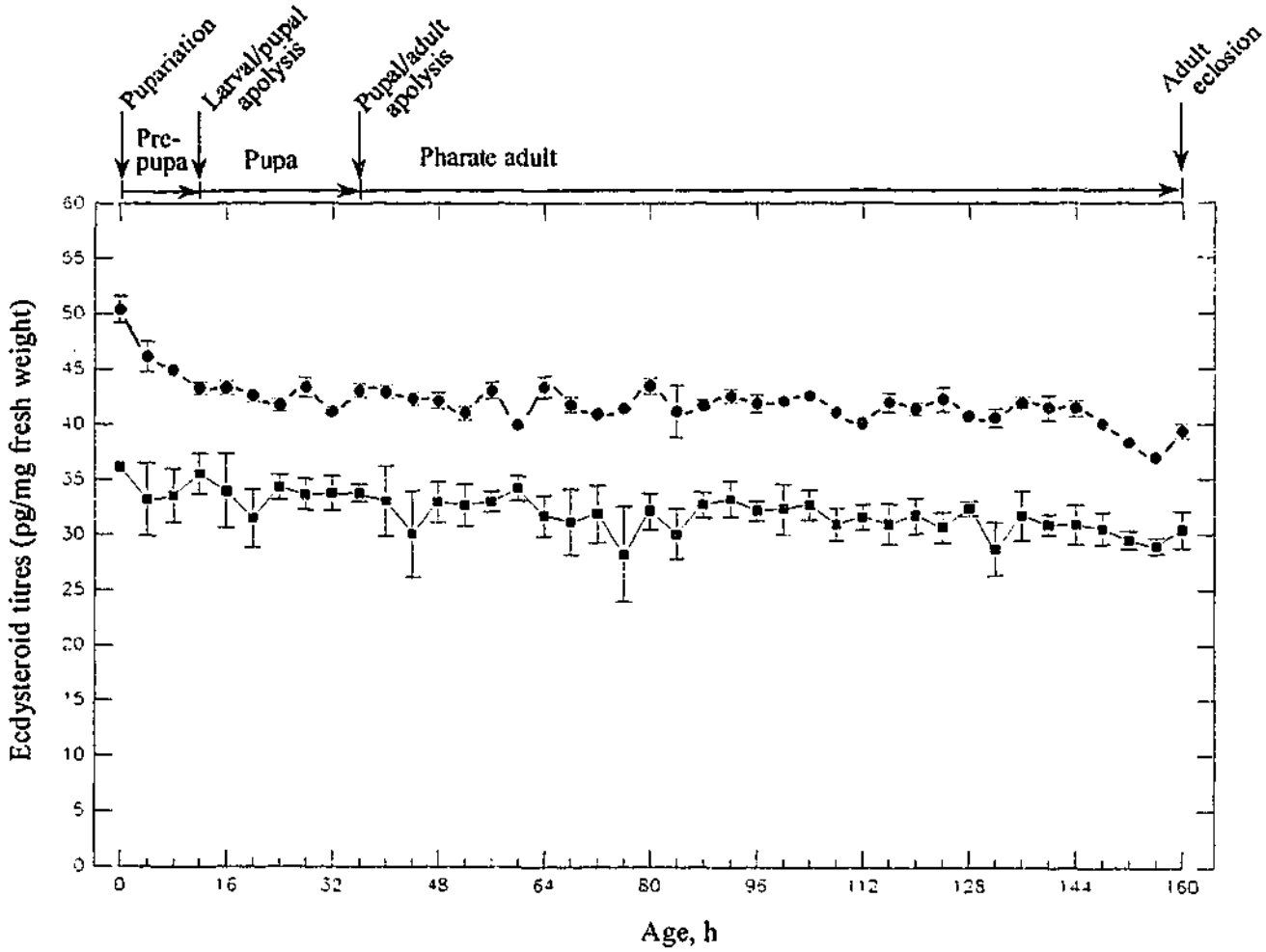


Fig. 32. Mean weight of females (solid circles •) and male (solid squares ■) prepupae, pupae and pharate adults of *Lucilia cuprina*. Each point represents the mean weight in mg ± SEM of 5 to 10 replicates, except where the SEM is too small to show.

3.3.2. Endocrinological aspects

The RIA measurement of whole-body ecdysteroid titres throughout prepupal, pupal and pharate-adult stages of *L. cuprina* is shown in Fig. 33 for females and Fig. 34 for the males. Each sample represents the ecdysteroid titre the mean ± SEM of 5 individuals. The details of the statistical analysis for t-Test, multiple comparisons of the mean values of ecdysteroid titres using one-way analysis of variance and the Tukey test of hypothesis are shown in Appendices 19 to 28.

3.3.2.1. Ecdysteroid titres during the prepupal stage

3.3.2.1.1. Ecdysteroids in female prepupae

As shown in Fig. 33, the level of ecdysteroids in females was 209.3 ± 8.1 pg/mg fresh weight at the formation of the WPP. The level then increased significantly to 281.2 ± 26.7 pg/mg 4 hours later (P=0.037, Appendix 19). High levels of ecdysteroids at this stage presumably initiated the larval/pupal apolysis which occurred 8 to 12 h after pupariation and were followed by developmental events summarised in Table 4. During the larval/pupal apolysis the ecdysteroid levels dropped significantly to 63.2 ± 2.0 pg/mg fresh weight (P=0) (Fig. 33).

3.3.2.1.2. Ecdysteroids in male prepupae

During pupariation, the level of ecdysteroids in males was 231.4 ± 27.2 pg/mg (Fig. 34). As in females, a significant increase then occurred 4 h later with a level of 346.1 ± 67.2 pg/mg (P=0.021, Appendix 20). This surge in ecdysteroid level is similar to that for females (described in Section 3.3.2.1.1). During the subsequent larval/pupal apolysis and concurrent pupal cuticle secretion, the ecdysteroid levels decreased to 73.4 ± 3.4 pg/mg (P=0.003).

Comparison between ecdysteroid in female and male prepupae using t-Test revealed that ecdysteroid levels were similar in prepupae of both sexes (P=0.153, Appendices 21 and 22).

3.3.2.2. Ecdysteroid titres during the pupal stage

3.3.2.2.1. Ecdysteroids in female pupae

By the time the pupa had formed, at 16 h after pupariation, the ecdysteroid levels had decreased to a level of 50.0 $\pm$ 2.7 pg/mg (Fig. 33). The levels then increased significantly (P=0, Appendix 23) and reached a level of 141.5 $\pm$ 31.2 pg/mg at 36 h after pupariation. This increase in ecdysteroid levels is likely to relate to initiation of pupal/adult apolysis which occurred at 40 h.

3.3.2.2.2. Ecdysteroids in male pupae

As in female pupae, sixteen hours following pupariation, ecdysteroid levels had dropped to 55.9 $\pm$ 1.6 pg/mg. But the level then increased significantly (P=0, Appendix 24) at 36 h after pupariation to a level of 193.3 $\pm$ 5.9 pg/mg (Fig. 34).

T-Test comparison during the pupal stage revealed that the ecdysteroid titre (at 36 h) in males was higher than that of females (P=0.005, Appendix 25)

3.3.2.3. Ecdysteroid titre during the pharate adult stage

3.3.2.3.1. Ecdysteroids in female pharate adults

After undergoing pupal/adult apolysis (36 h) and entering the pharate adult stage at 40 h, the level of ecdysteroids in the female continued to increase and peaked (P=0.0002) between 60 and 68 h with a maximal value of 710.5 ± 44.6 pg/mg at 68 h (Fig. 33, Appendix 26). The levels then decreased to a minimum value of 249.4 ± 15.4 pg/mg at 88 h (P=0.0002). A second surge in ecdysteroid level occurred 4 hours later with a maximum of 646.8 ± 20.6 pg/mg at 92 h. This second peak dropped relatively rapidly (over 8 h) to a low level of 212.4 ± 12.6 pg/mg at 104 h (P=0.0002). The level then continued to decrease until the time when adult flies started to emerge, at approximately 160 h after pupariation (P=0.0001). At adult emergence the level of ecdysteroids was low: 47.8 ± 6.9 pg /mg. The developmental events associated with these two peaks during the pharate-adult development are summarised in Table 4 (discussed later).

3.3.2.3.2. Ecdysteroids in male pharate adults

As in females, ecdysteroids levels in male pharate adults continued to increase after the pupal/adult apolysis (at 36 h), but this peak reached its maximal level of 764.4 ± 25.3 pg /mg and at 56 h (P=0.0002, Appendix 27). This is much earlier than in females (Fig. 34). The levels subsequently decreased to 211.8 ± 8.1 pg/mg at 88 h (P=0.0002) as in females. In addition, a second significant peak occurred at the same time as in females (92 h) with a level of 581.26 ± 51.94 pg /mg (P=0.0007), which then dropped to 198.5 ± 13.3 pg/mg at 104 h. The levels then continued to decrease until the time when the adult flies started to emerge, at

approximately 160 h after pupariation (P=0.0001). At emergence, the level of ecdysteroids in adult males was 43.5 $\pm$ 5.8 pg/mg.

Comparison between female and male ecdysteroid levels during the pharate adult stage using a t-Test showed no significant difference (Appendix 28). The maximal values of ecdysteroid titres reached in the pharate adults of both sexes did not differ between sexes either (P=0.1307 for the first peak and P=0.1160 for the second one, Appendix 29). Interestingly, the first peak occurred approximately 12 h earlier in pharate males than in females, suggesting a possible role in sexual or reproductive development. In contrast, the second peak occurred exactly at the same time in both sexes, suggesting a role in non-sexual development and presumably in the development of the imaginal disks to form adult internal organs.

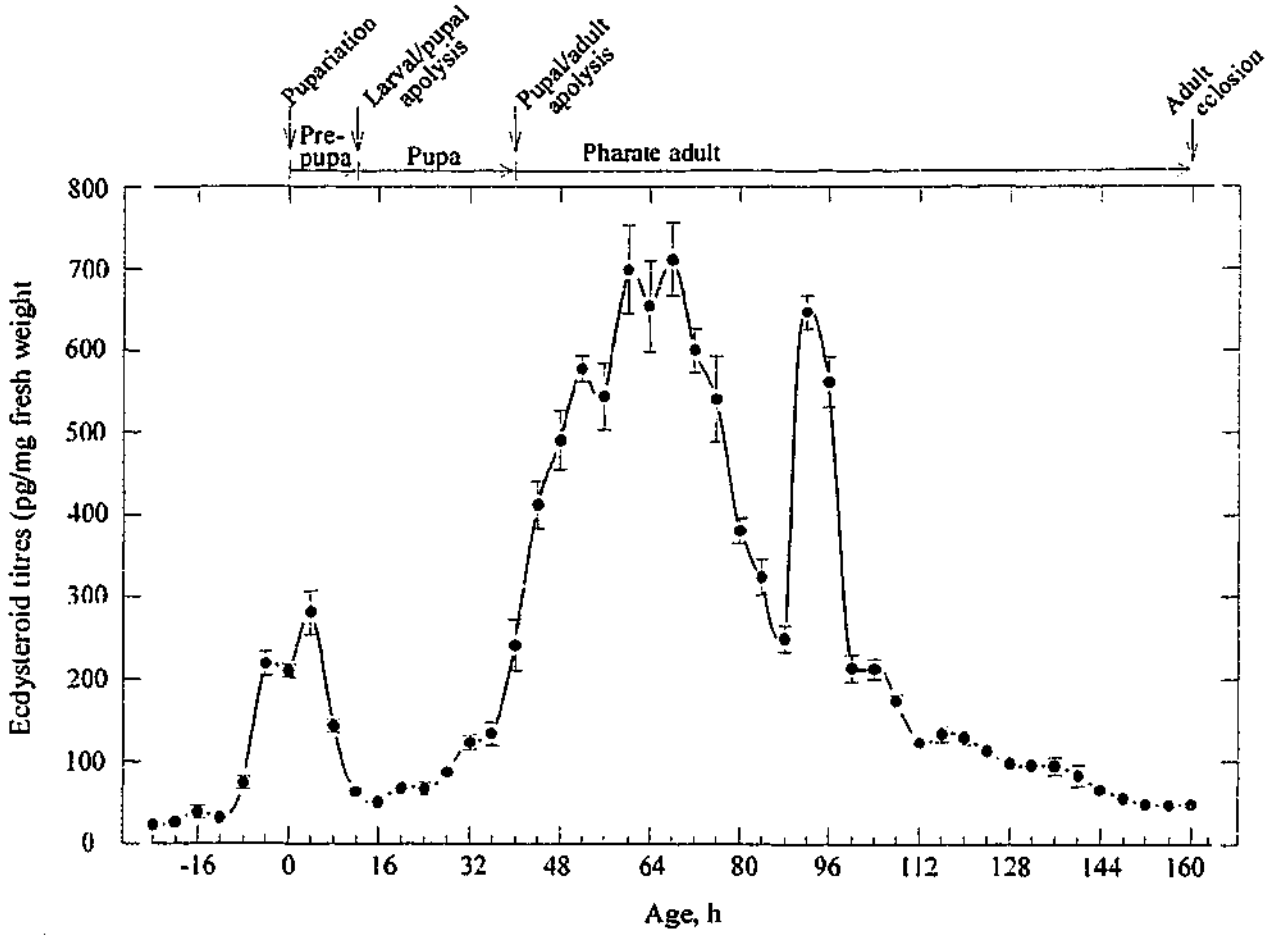


Fig. 33. Whole-body ecdysteroid titres throughout prepupal, pupal and pharate adult stages of development in the females of *Lucilia cuprina* reared at 27° C quantified by RIA. Each point represents the mean titre expressed in pg ecdysteroids/mg fresh body weight ± SEM of 5 individuals, except where the SEM was too small to show.

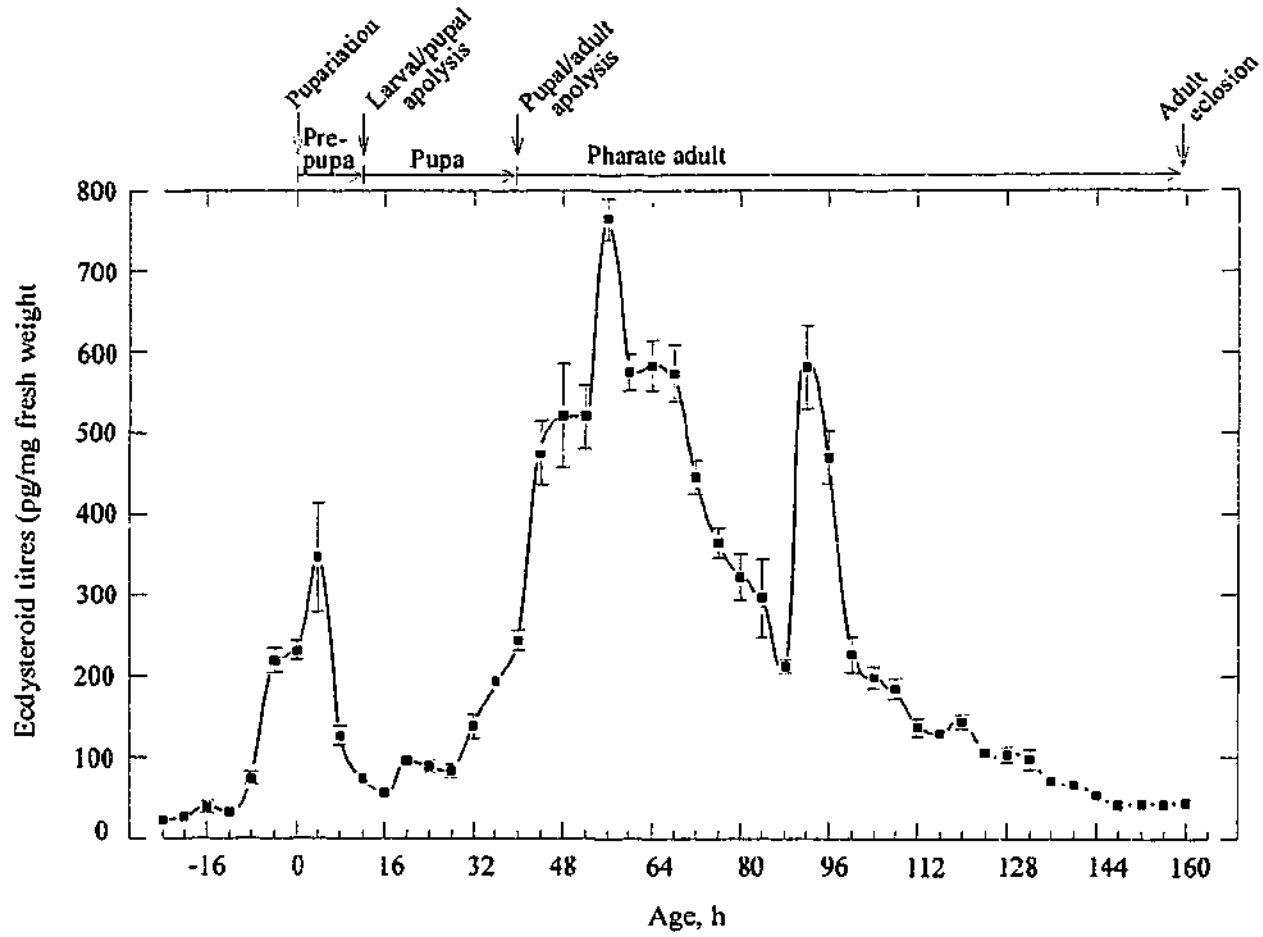


Fig. 34. Whole-body ecdysteroid titres throughout prepupal, pupal and pharate adult stages of development in the males of *Lucilia cuprina* reared at 27° C quantified by RIA. Each point represents the mean titre expressed in pg ecdysteroids/mg fresh body weight ± SEM of 5 individuals, except when too small to show.

Table 4. A summary of the time course of development and ecdysteroid levels throughout prepupal, pupal, and pharate-adult of *Lucilia cuprina* reared at 27° C showing coincident developmental events reported in this thesis, as well as data from the literature for *L. cuprina* and related species.

Age (h)	Ecdysteroid levels	Stage of development	Developmental event	Reference for comparison
0		White prepupa	**Pupariation**	
			Increase in granular haemocytes	
			Epidermal cells enlarge	
		Prepupa	Muscles are still striated	
1			Sclerotization of the puparium	
4	Prepupal peak		Disappearence of muscle striations	Barritt and Birt (1971)
8 - 12			**Larval/pupal apolysis**	
			The stage of cryptocephalic pupa	Fraenkel and Bhaskaran (1972)#
			Secretion of the pupal cuticle	Carruthers and Roberts (1979)*
			Formation of the discs of the imaginal thoracic structures	
16	Low		Histolysis of larval muscles	
			The pupal cuticle is visible 1 - 2 μ separate from the epidermis of the thorax and its appendages	
		Pupa		
			Breakdown of the anterior fat body cells and the basement membrane enclosing the fat body	Barritt and Birt (1971)
24 - 28			Head evagination forms the phanerocephalic pupa	Fraenkel and Bhaskaran (1972)#
	Rapid increase		Cuticular expansion, retraction of larval tracheae, migration of brain, tracheae, fat body into the newly formed imaginal structure	Zdarek and Friedman (1986)#
36 - 40			**Pupal/adult apolysis**	
56	First pharate adult peak (male)		Thoracic and leg muscles are growing rapidly	
			Formation of flight muscles	
68	First pharate adult peak (female)		Eye pigmentation	
		Pharate adult	Leg differentiation into segments with setae and hair and claws on the feet.	Barritt and Birt (1971)
72				
92	Second pharate adult peak		Endocuticle deposition	Whitten (1969)#
120			Definitive adult morphology	Barritt and Birt (1971)
160-184	Gradual decrease		**Adult eclosion**	

* Refers to the flesh fly *Tricholioproctica impatiens*
\# Refers to the flesh fly *Neobellieria bullata*

3.4. Adult stage of development

In order to study the role of ecdysteroids in regulating developmental processes, such as gonad development and the onset of the female reproductive cycle during the adult stage of *L. cuprina*, females and males were examined separately and developmental events related to recorded ecdysteroid titres. In addition, since oogenesis in wild-type *L. cuprina* is apparently dependent on a protein meal (Barton Browne *et al.*, 1976), the effect of protein was analysed.

3.4.1. Developmental aspects

3.4.1.1. Female whole-body weights

As shown in Fig. 35, the mean weight of females at eclosion was 36.07 ± 6.42 mg (± SEM). Body weight appeared to slightly decrease to 32.82 ± 0.98 mg, within the next 8 h, but this was not statistically significant (P>0.05, Appendices 30 and 31). Females fed only sugar and water from emergence did not show significant body weight increase until at least 116 h of age (Fig. 35, open circles). Mean body weight of females showed some fluctuation, presumably, related to the amount of sugar and water taken at the time of measurement, but these were not statistically significant (P>0.05, Appendices 30 and 31). Furthermore, no clear pattern of decrease/increase in body weight could be distinguished between photophase and scotophase (Fig. 35).

In contrast, in liver-fed females, the mean body weight increased significantly (P=0, Appendix 31) within less than 1 h after liver ingestion and reached 49.26 ± 1.61 mg within 4 h of the first protein meal (at 27 h, Fig. 35, solid circles). Thereafter, the body weight remained relatively constant (P>0.05,

Appendix 31) until at least 116 h of age. The maximal value of 53.36 ± 0.51 mg was reached 68 h following eclosion (Fig. 35). As in non-protein fed females, no clear pattern of variation could be detected in animals sampled during photophase or scotophase.

In conclusion, no significant body weight increase could be detected in non-protein fed females. A comparison between protein-fed and non-protein fed females indicates that protein ingestion increased the mean weight of adult females significantly (t-Test, P=0, Appendix 32).

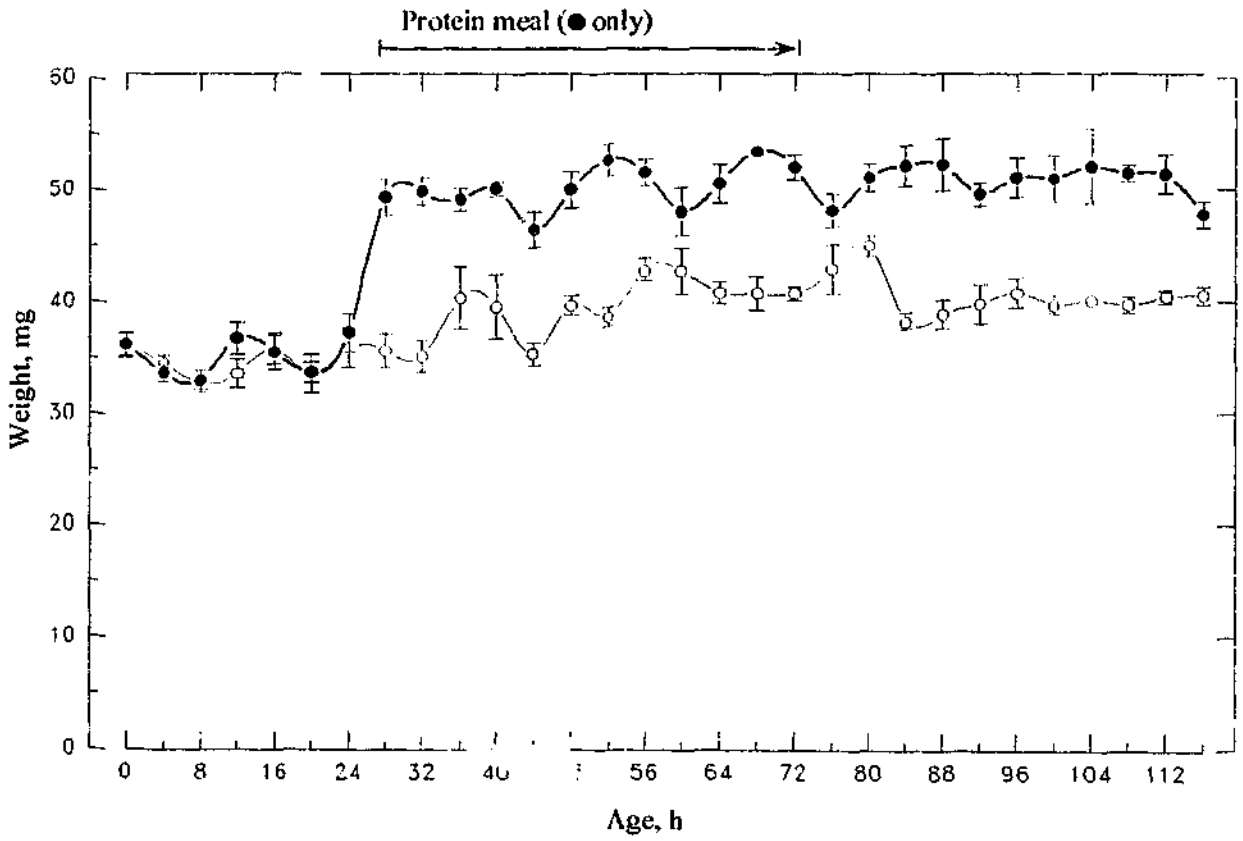


Fig. 35. Mean body weights of the adult females of *Lucilia cuprina* fed different diets. Solid circles (●) represent females fed sugar, water and *ad libitum* lamb liver for 48 h starting at 27 h (arrow) after eclosion. Open circles (o) represent females fed sugar and water only. Each point represents the mean value ± SEM (except where too small to show) of 5 individuals. Shaded areas indicate the scotophase.

3.4.1.2. Weight of the female reproductive system

To identify the contribution of egg development in the body weight increase of protein-fed females, the ovaries, oviducts and associated reproductive organs were dissected out and weighed during the first ovarian cycle. The females were given access to a protein meal at 27 h following eclosion. Preliminary observations showed that yolk deposition started at 52 h, and as a result measurements were started at 48 h post eclosion.

Up to 72 h post-eclosion no significant increase in weight of the female reproductive system could be detected (from 2.94 ± 067 mg at 48 h to 6.10 ± 0.99 mg at 72 h) (Fig. 36). After 72 h post-eclosion (*i.e.* 45 h after the first protein meal) the reproductive system weight increased dramatically to a maximum of 13.67 ± 0.83 mg at 84 h (P=0; Appendix 33). During the next hours the mean weight remained relatively constant until 96 h, by which time the flies were competent to lay eggs.

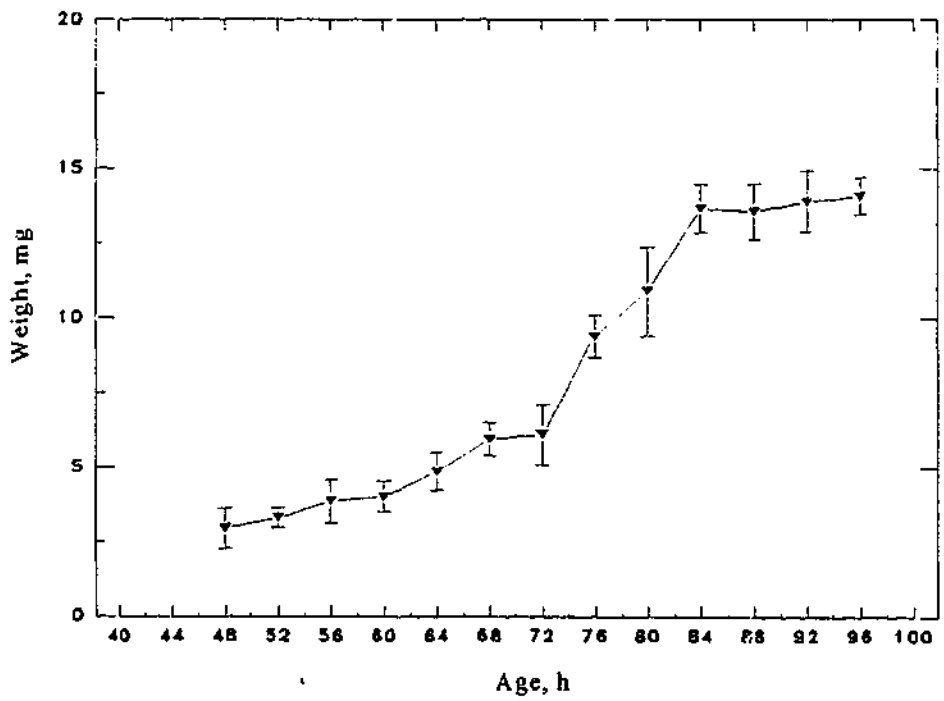


Fig. 36. The mean weights of reproductive systems dissected out from females of *Lucilia cuprina* fed sugar, water and *ad libitum* lamb liver for 48 h starting at 27 h after eclosion. Each point represents the mean weight ± SEM of 5 replicates, each of which consists of one pair of ovaries, the oviducts and associated organs.

3.4.1.3. Weights of female bodies without reproductive systems

The mean weight of the female bodies after the reproductive system had been removed was 44.71 ± 1.22 mg at 48 h (Fig. 37). The weight did not decrease significantly up to 72 h post-eclosion. It was only thereafter that a significant decrease in weight occurred down to 33.57 ± 2.51 mg at 76 h (P=0.024; Appendix 34). From 76 h onwards the mean weight remained relatively constant.

In conclusion, the results presented in Figs. 36 and 37 clearly demonstrate that body weight increase of females after protein ingestion is solely caused by an increase in the weight of the reproductive system.

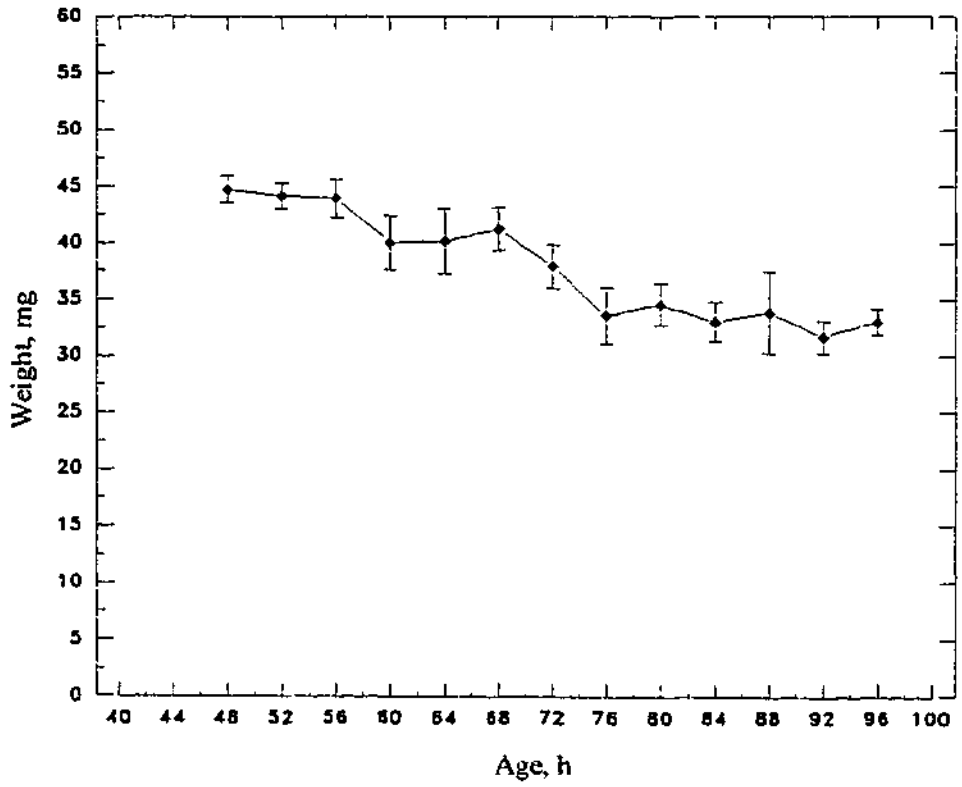


Fig. 37. Mean body weight minus the reproductive system of adult females of *Lucilia cuprina* fed sugar, water and *ad libitum* lamb liver for 48 h starting at 27 h. Each point represents the mean weight ± SEM of 5 replicates.

3.4.1.4. Ovarian development during the first reproductive cycle

The developmental events occurring during the first female reproductive cycle of protein-fed females are summarised in Table 5 and Fig. 38. Examination of the stages of the ovarian development started at 48 h, at which time 2 out of 5 females examined had developed secondary follicles (Stage I). Yolk deposition was first observed at 52 h post-eclosion with the yolk occupying less than one third of the egg (Stage II, Fig. 38). By 76 h all flies showed eggs in which the yolk occupied 50 % - 75 % of the egg and the nurse cells started to degenerate (Stage III). At 80 h following eclosion, all flies had completed yolk deposition (Stage IV), and by 96 h fully matured eggs had developed as indicated by the yolk occupying the entire egg. At this stage the hatching pleat was clearly visible (Stage V).

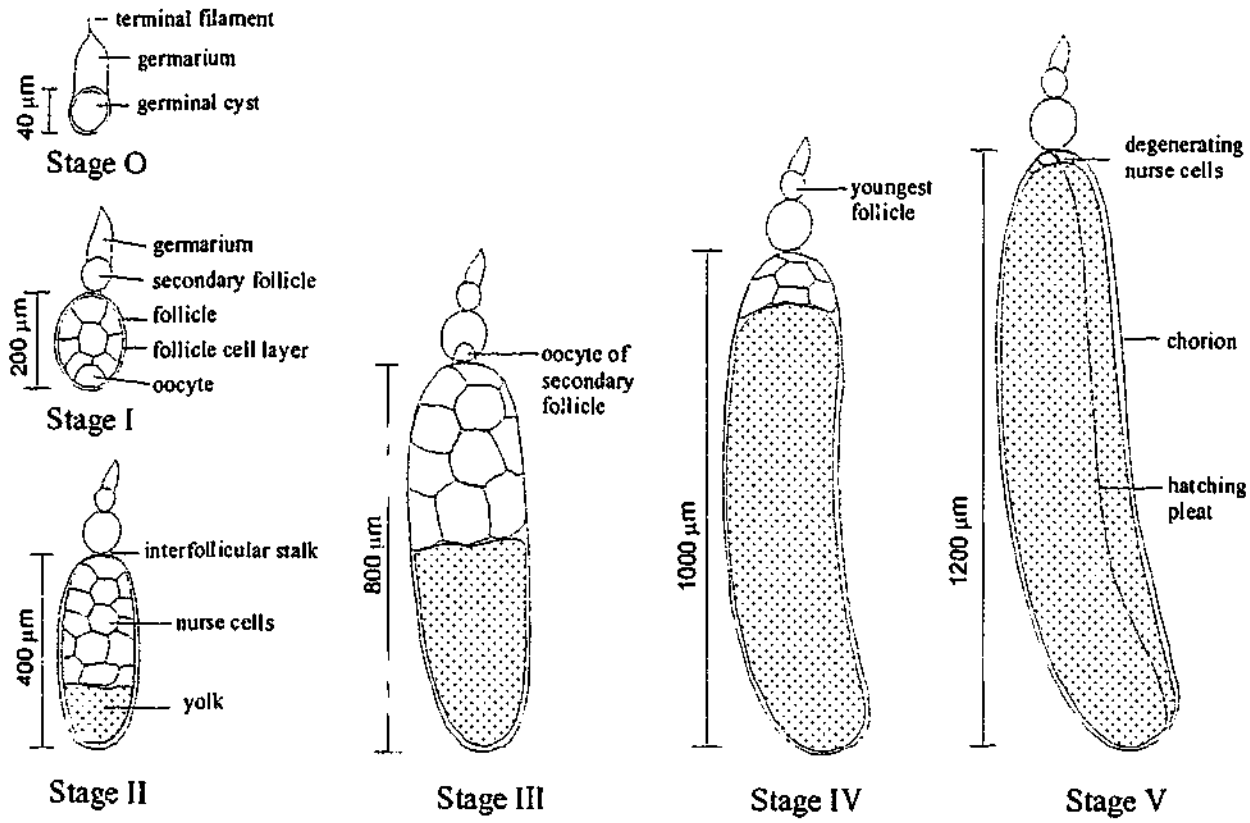


Fig. 38. Developmental stages (0 - V) of the egg in the ovary of *Lucilia cuprina* during the first reproductive cycle. Redrawn after Vogt *et al.* (1974).

Table 5. A summary of developmental events and ecdysteroid levels during the first female reproductive cycle of *Lucilia cuprina* reared at 27° C fed with sugar, water and *ad lib* lamb liver for 48 h starting 27 h after eclosion.

Age (h)	Ecdysteroid levels	Diet	Developmental event	Reference
0	Low	Sugar + water	**Eclosion**	
27			First ovarian follicle is formed but not completely separated from germarium	
48		Protein meal	Second follicle is produced and the first becomes elongated	
52			Start of yolk deposition	
68	Peak		Oocytes are clearly distinguished in unstained whole mounts	
76			Nurse cells start to degenerate	
80	Low		Yolk deposition is completed, followed by deposition of the chorion	Clift and McDonald (1973)
			Third follicle is produced	Vogt, *et al.* (1974)
96			Chorion deposition is completed	
97			**Oviposition of the first follicle**	

3.4.1.5. Male whole-body weights

Figure 39 shows that the mean weight of entire males which was 30.32 ± 1.72 mg at eclosion, decreased to 23.26 ± 1.09 mg within the next 8 h (P=0.011, Appendix 35), following the same trend as in females before liver was supplied. Within 1 h after the liver was supplied, the body weight of males started to increase significantly up to a maximum of 32.36 ± 1.21 mg at 32 h post-eclosion (P=0, Appendix 35). However, 21 h after the liver meal, the weight of adult males was not significantly different from the weight before a liver meal (P>0.05, Appendix 35). Thereafter the mean weight of males remained relatively constant and no significant variations could be detected up to 116 h following eclosion, at

which time the weight was 24.7 ± 1.25 mg (Fig. 39). Interestingly, male weight displayed a decreasing trend during scotophases (Fig. 39), which could indicate that feeding may not take place, or at least decrease, at night. Presumably, due to the small number of replicates, however this trend could not be shown statistically ($P>0.05$, Appendix 35).

In addition, the results shown in Fig. 39 indicate that the mean body weights of adult males fed liver (from 0900 h on day 2, for 48 h) were significantly lower than those of the females from eclosion onward and at least 116 h of age (t-Test, P=0, Appendix 36).

In conclusion, the liver meal resulted in a rapid increase in body weight of males within 5 h of protein ingestion, but no weight fluctuations were observed thereafter.

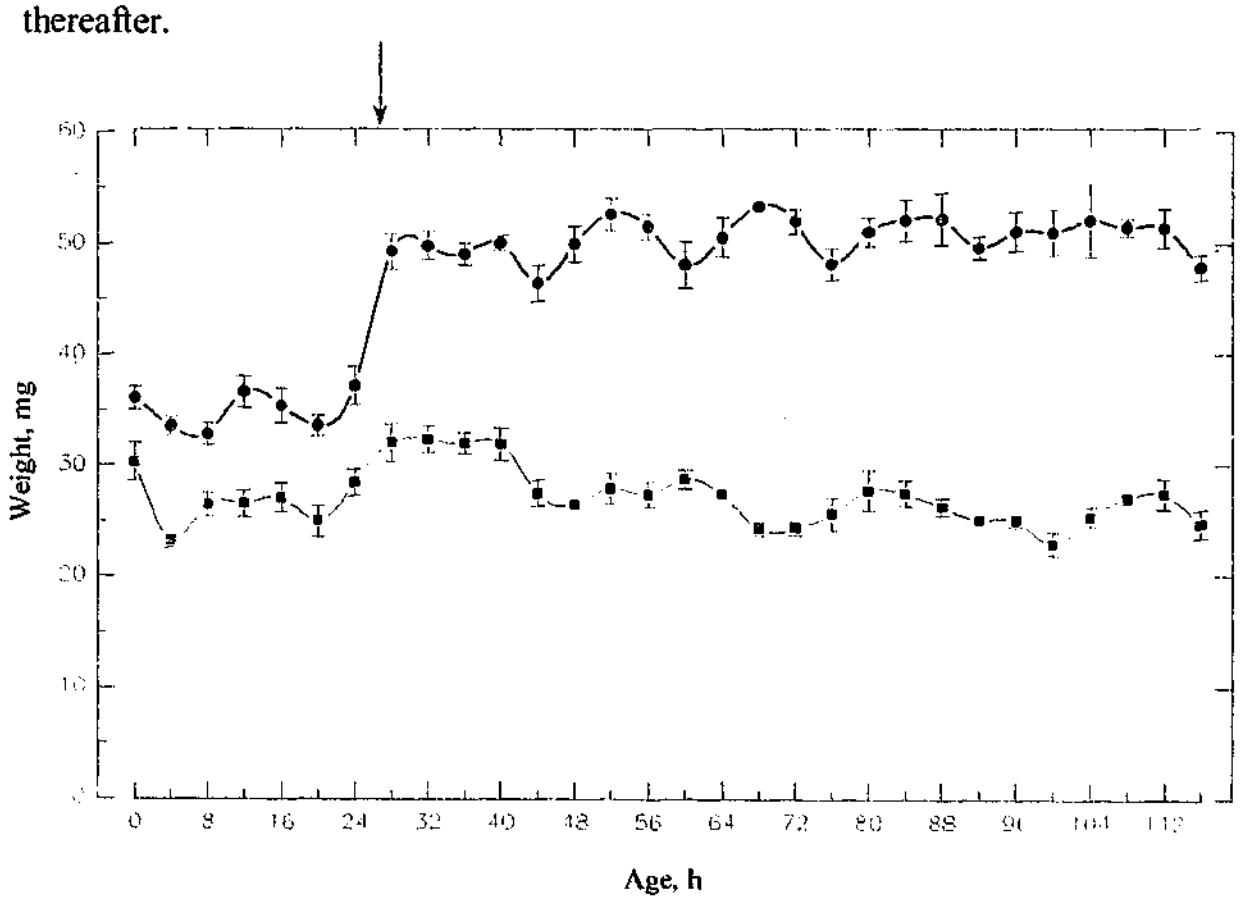


Fig. 39. The mean body weight of male (solid squares, ■) and female (solid circles, ●) adults of *Lucilia cuprina* fed with sugar, water and *ad libitum* lamb liver for 48 h starting at 27 h (see arrow) after eclosion. Each point represents the mean value ± SEM of 5 replicates, except when too small to show. Shaded areas indicate the scotophase.

3.4.2. Endocrinological aspects

3.4.2.1. Ecdysteroid titres in liver-fed adult females

3.4.2.1.1. Whole body ecdysteroid titres

Results shown in Fig. 40 indicate that the level of ecdysteroids at eclosion was relatively high (10.17 ± 3.41 pg/mg fresh weight) and then decreased significantly to a low minimum of 1.69 ± 0.23 pg/mg fresh weight at 28 h after eclosion (P=0, Appendix 37). The flies were given *ad lib.* liver at 27 h for 48 h and, the ecdysteroid titres remained relatively constant until 48 h post-eclosion (Fig. 40). Thereafter, coinciding with the formation of the secondary follicle and the start of yolk deposition, the ecdysteroid levels increased rapidly and a significant peak was reached 68 h after eclosion (P=0) with a value of 40.45 ± 12.33 pg/mg fresh weight. This peak occurred during the active phase of yolk deposition in the eggs (stages II and III, Fig. 38). Within the next 12 hours the ecdysteroid titres had dropped (P=0) to a low value of 4.49 ± 0.44 pg/mg fresh weight at 80 h (P=0), which corresponds to the end of vitellogenesis (stage IV, Fig. 38). Thereafter the titres remained relatively constant throughout the stage up to 116 h (Fig. 40, Appendix 37).

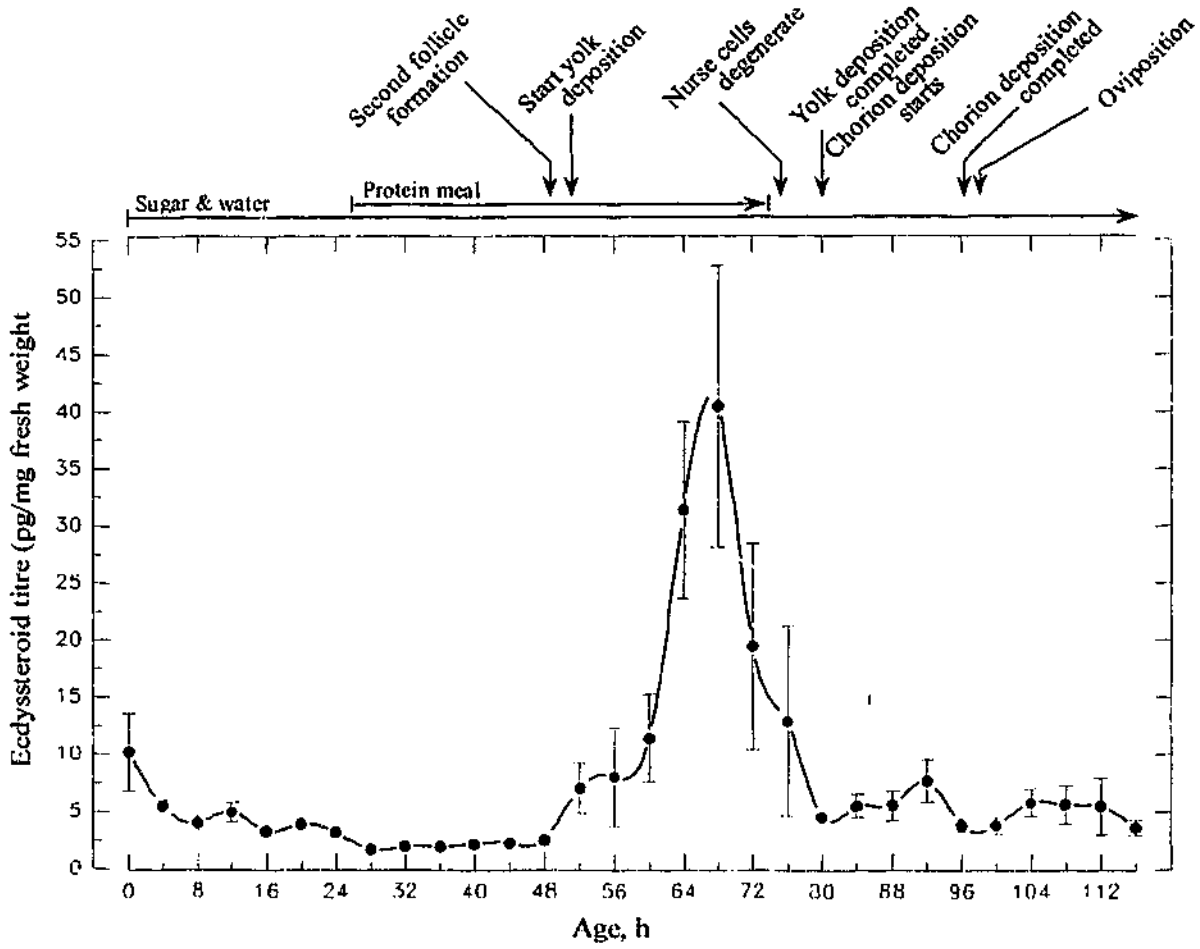


Fig. 40. Ecdysteroid titres measured by RIA in whole bodies of female adults of *Lucilia cuprina* reared at 27° C and fed with sugar, water and *ad libitum* lamb liver for 48 h starting at 27 h after eclosion. Each point represents the mean value ± SEM, (except when too small to show) of 5 whole-body replicates. Shaded areas indicate the scotophase.

3.4.2.1.2. Ecdysteroid titre in the female reproductive system

In order to check whether the reproductive system contributed to the high ecdysteroid levels in whole-body homogenates of liver-fed females, observed at 68 h (Fig. 41), titres were measured individually by RIA in the combined ovaries, oviducts and associated organs. Details of the statistical analysis are presented in Appendix 38.

The measurement of ecdysteroid titres in the reproductive system was commenced at 48 h after eclosion, or 21 h after the first protein meal, and revealed that at this time the level was 7.81 $\pm$ 1.31 pg /mg fresh weight, which coincided with the formation of the secondary follicles (Stage I, Fig. 38). A rapid increase occurred after the start of yolk deposition at 52 h and during the following stages until a maximum was reached at 68 h after eclosion (Fig. 41: 128.26 $\pm$ 16.09 pg, P=0). The ecdysteroid level then dropped over the next 16 h to a minimum of 16.13 $\pm$ 4.66 pg at 84 h (P=0) only a few hours after the yolk deposition was completed. During the rest of the stage the ecdysteroid levels remained relatively constant until 96 h. The apparent increase seen between 84 and 96 h in Fig. 41 was not significant (Appendix 38).

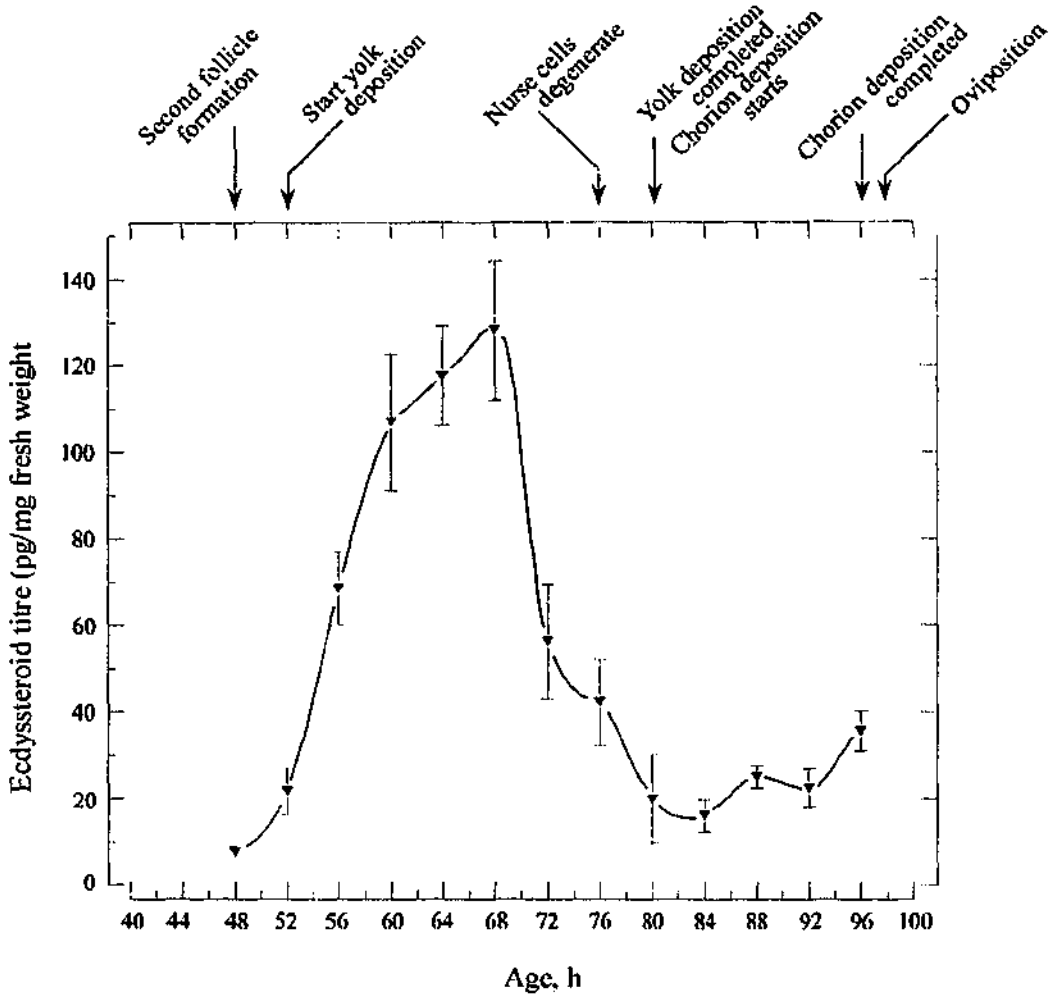


Fig. 41. Ecdysteroid titres measured by RIA in female reproductive systems (ovaries, oviducts and associated organs), dissected from female adults of *Lucilia cuprina* reared at 27° C and fed with sugar, water and *ad libitum* lamb liver for 48 h starting 27 h after eclosion. Each point represents the mean value ± SEM of 5 replicates, except where too small to show. Shaded areas indicate the scotophase.

3.4.2.1.3. Ecdysteroid titres in the female body without the reproductive system

Figure 42 shows the level of ecdysteroid titres in the bodies of liver-fed females from which the ovaries, oviducts and associated organs had been removed. The details of the statistical analysis are presented in Appendix 39.

Overall, the level of ecdysteroids in the bodies without reproductive system was very low. At 48 h post-eclosion the titre was only 1.77 ± 0.69 pg/mg fresh weight. The levels then increased slowly to reach a maximal level of 12.92 ± 3.16 pg at 64 h (P=0.025). During the rest of the developmental period, the levels decreased continuously to a minimum of 1.94 ± 1.02 pg at 96 h after eclosion (P=0.029).

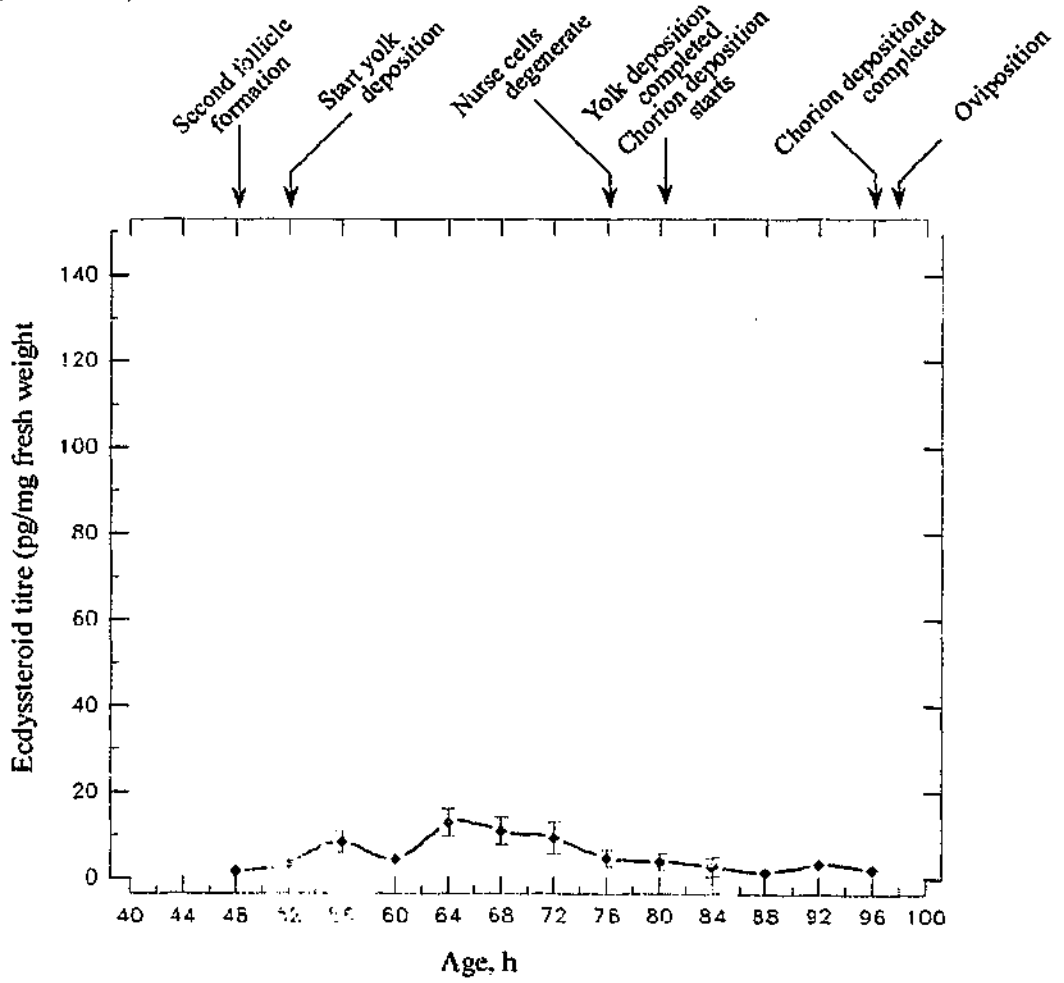


Fig. 42. Ecdysteroid titres measured by RIA in female adult bodies without their reproductive systems in *Lucilia cuprina* reared at 27° C, fed sugar, water and *ad libitum* lamb liver for 48 h starting at 27 h after eclosion. Each point represents the mean value ± SEM of 5 replicates except where too small to show. Shaded areas indicate the scotophase.

3.4.2.1.4. Summary of ecdysteroid titres in protein-fed females

Fig. 43 presents a summary of results for ecdysteroids for female adults reared on sugar, water and *ad lib.* lamb liver. The graph shows the relationship between whole body, carcass, and reproductive tract. The figure below clearly demonstrates that ecdysteroids in ptotein-fed females are present mainly in the reproductive tract. During the first female reproductive cycle, only one major peak of ecdysteroid titres was observed at 68 h in whole bodies (solid circles and Fig. 40) and this was found to be confined to the reproductive system (solid triangles and Fig. 41). Ecdysteroid levels in bodies from which reproductive systems had been removed were very low and increased slowly only to 64 h post-eclosion (solid diamonds and Fig. 42).

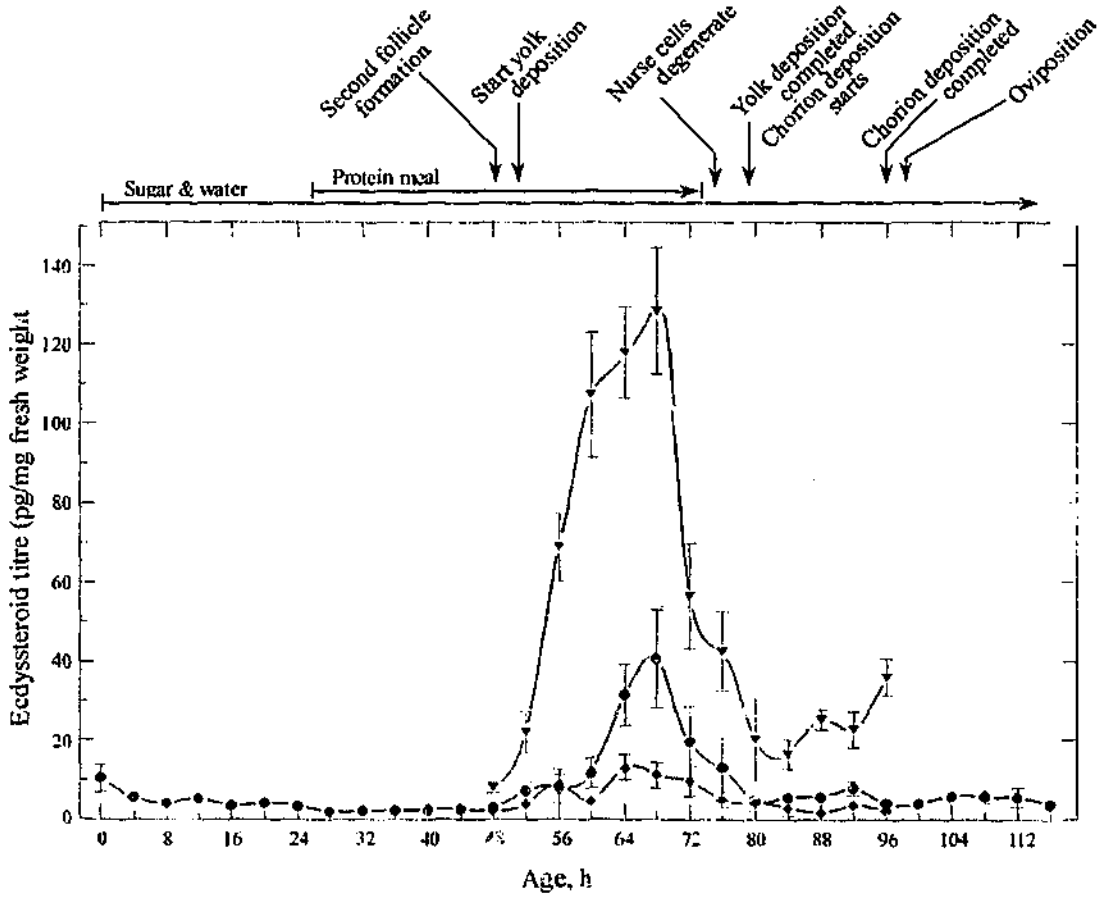


Fig. 43. A summary of ecdysteroid titres measured by RIA in female adults of *Lucilia cuprina* reared at 27° C and fed with sugar, water and *ad libitum* lamb liver for 48 h starting at 27 h after eclosion (solid circles •), in female reproductive systems (ovaries, oviduct and associated organs) (solid triangles ▾) and in female adult bodies without reproductive system (solid diamonds ♦). Each point represents the mean value ± SEM of 5 replicates except where too small to show. Shaded areas indicate the scotophase. This clearly demonstrates that ecdysteroids are present mainly in the reproductive tract in adult females.

3.4.2.2. Ecdysteroid titres in adult females fed solely sugar and water

The profile of ecdysteroid titres in whole bodies of non-protein-fed females is shown in Fig. 44, and details of the statistical analysis are presented in Appendix 40.

At eclosion the level of ecdysteroids in the non-protein fed females was 9.07 ± 0.15 pg/mg fresh weight (Fig 45). The titres decreased rapidly to a minimum value of 3.10 ± 0.15 pg /mg fresh weight at 16 h (P=0) and remained low and constant until at least 116 h after eclosion (P>0.05).

Ecdysteroid titres in non-protein fed females were thus very low throughout the developmental period of 116 h, in which barely any variations were detected. It is noteworthy that no ovarian development occurred during this period.

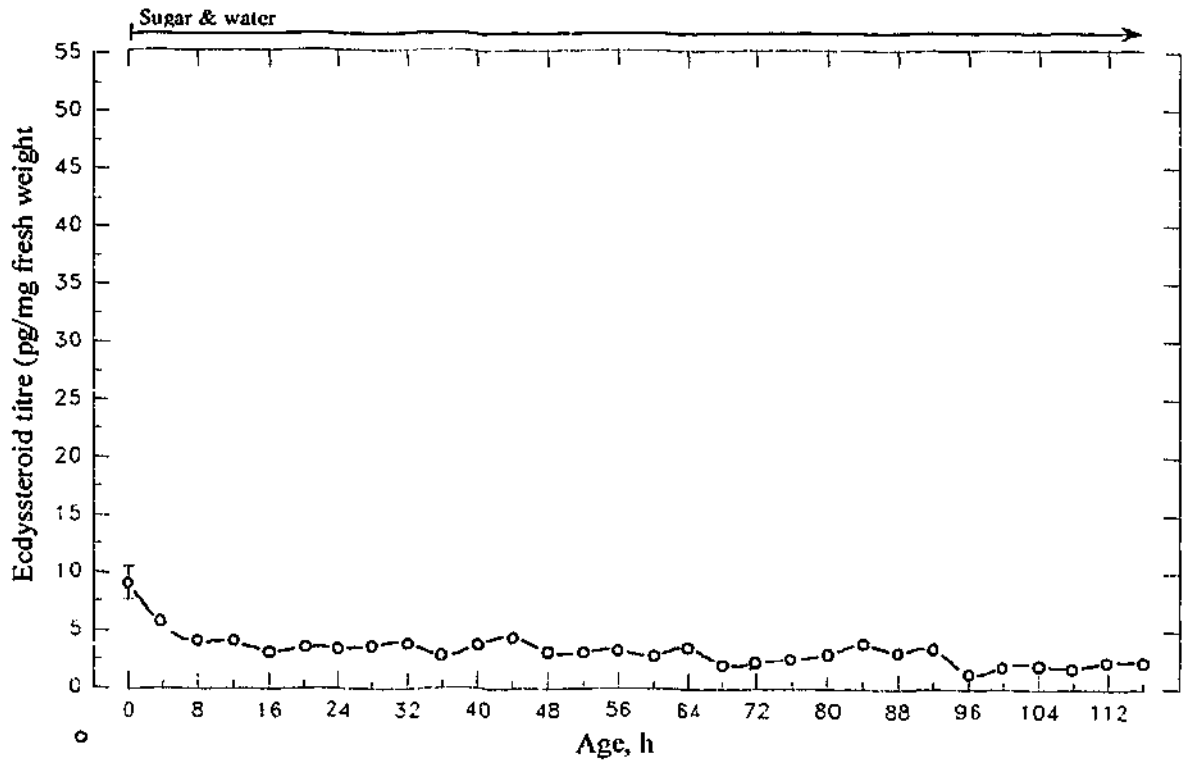


Fig. 44. Ecdysteroid titres measured by RIA in female adults of *Lucilia cuprina* reared at 27° C and fed only sugar and water. Each point represents the mean value ± SEM of 5 whole-body replicates, except where too small to show. Shaded areas indicate the scotophase.

3.4.2.3. Effect of protein ingestion on ecdysteroid levels in females

A comparison between ecdysteroid titres in protein-fed and non-protein fed adult females of *L. cuprina* is shown in Fig. 45. Statistical analysis using t-Test for these two groups of animals revealed that the levels of whole-body ecdysteroids in protein-fed females were significantly higher than those of non protein-fed flies (P=0, Appendix 41).

The data in Fig. 45 show clearly that protein ingestion is responsible for the tremendous increase in ecdysteroid titres in females after a protein meal. As emphasised in section 3.4.2.1.4 and Fig. 43, that most ecdysteroids in protein-fed females are located in the reproductive system. This suggests that most ecdysteroids in adult females are confined to developing eggs. The possible relation between protein uptake, high ecdysteroid titres and egg development will be discussed later.

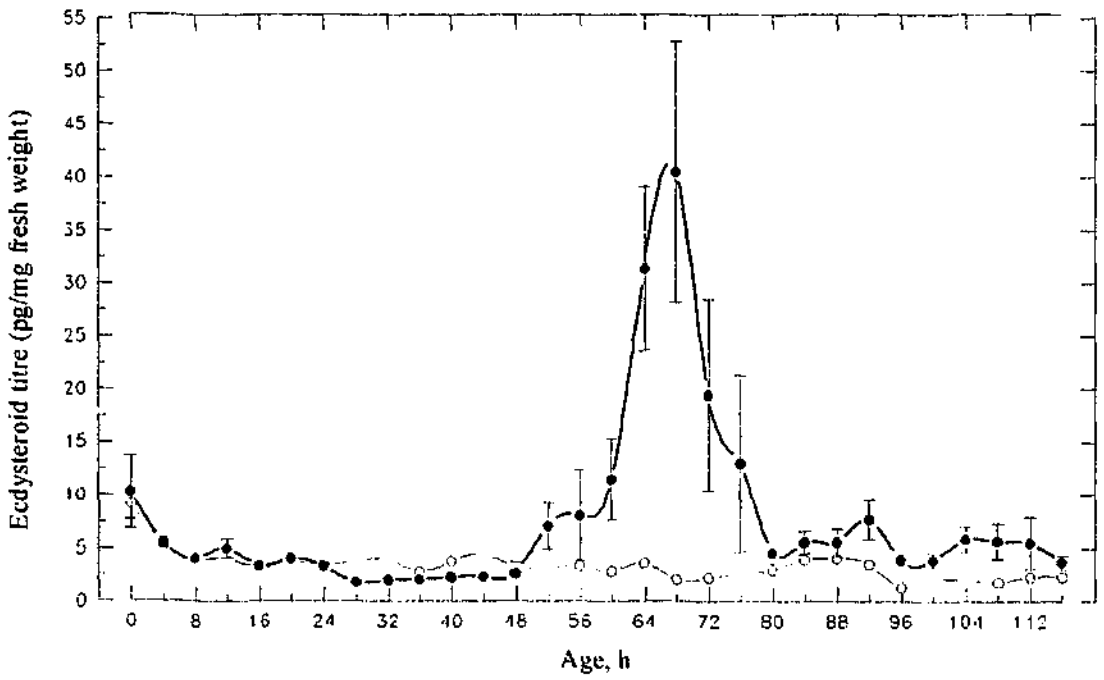


Fig. 45. A comparison of ecdysteroid titres measured by RIA between female adults of *Lucilia cuprina* reared at 27° C and fed with sugar, water and *ad libitum* lamb liver (solid circles) for 48 h starting at 27 h after eclosion and in female adults fed only sugar and water (open circles). Each point represents the mean value ± SEM of 5 whole-body replicates, except where too small to show. Shaded areas indicate the scotophase. This clearly shows that protein ingestion is responsible for the tremendous increase in ecdystroid levels in females.

3.4.2.4. Ecdysteroid titres in adult males

Figure 46 shows the levels of whole body ecdysteroid titres in protein-fed adult males. The details of the statistical analysis are presented in Appendix 42. In newly emerged male adults, the level of ecdysteroids was 10.78 ± 3.12 pg/mg fresh weight, which decreased until to a value of 2.26 ± 0.33 pg/mg fresh weight 28 h after eclosion (P=0). After being given a protein meal at 27 h males did not show significant detectable increase in whole-body ecdysteroids values, which remained relatively constant up to 116 h after eclosion. In conclusion, no effect of protein ingestion could be detected on ecdysteroid profiles of males.

Interestingly, the same pattern of ecdysteroid profiles is found in males as in non-protein fed females: relatively high levels at eclosion followed by a decrease within 16 h and an almost undetectable level for the rest of the developmental period (until 116 h) (Fig. 44 and 46). A comparison of ecdysteroid titres between protein-fed females and males of *L. cuprina* (Fig. 47), on the other hand, revealed that the levels of ecdysteroids in protein-fed females were significantly higher (P=0) than those in male flies (Appendix 43).

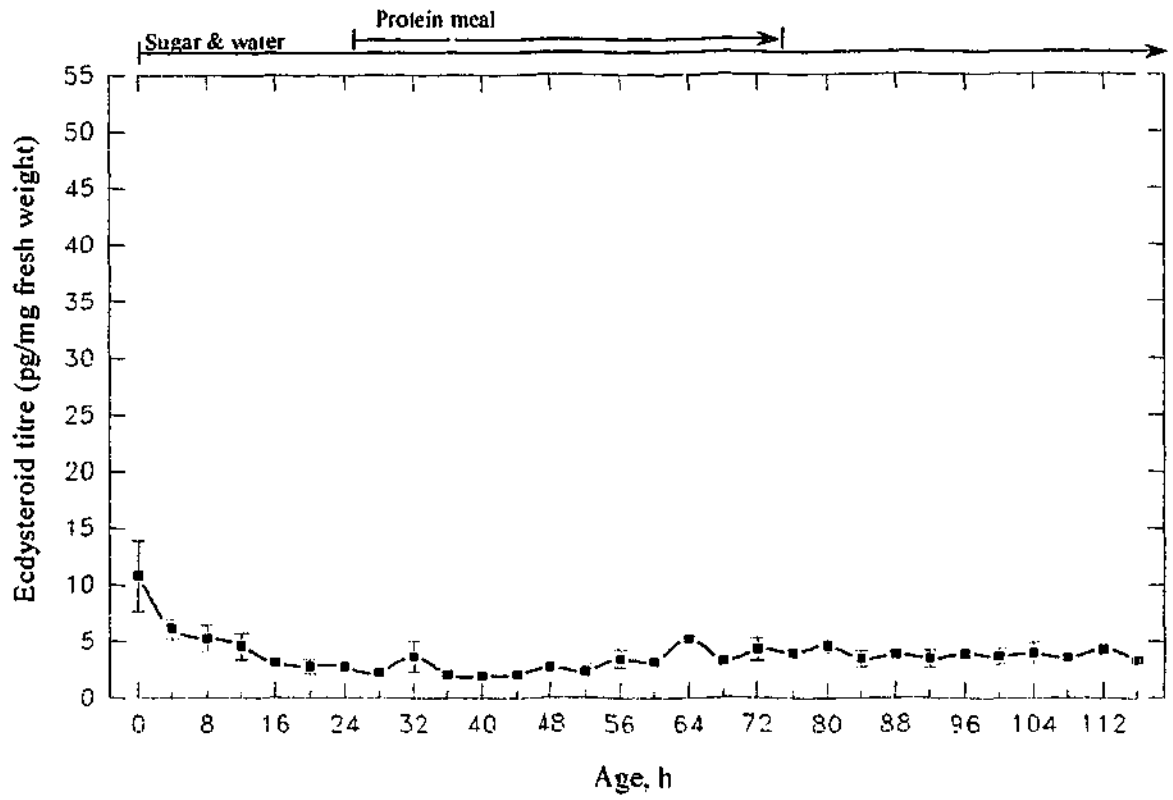


Fig. 46. Ecdysteroid titres measured by RIA in male adults of *Lucilia cuprina* reared at 27° C and fed with sugar, water and *ad libitum* lamb liver for 48 h starting at 27 h after eclosion. Each point represents the mean value ± SEM (except where too small to show) of 5 whole-body replicates. Shaded areas indicate the scotophase.

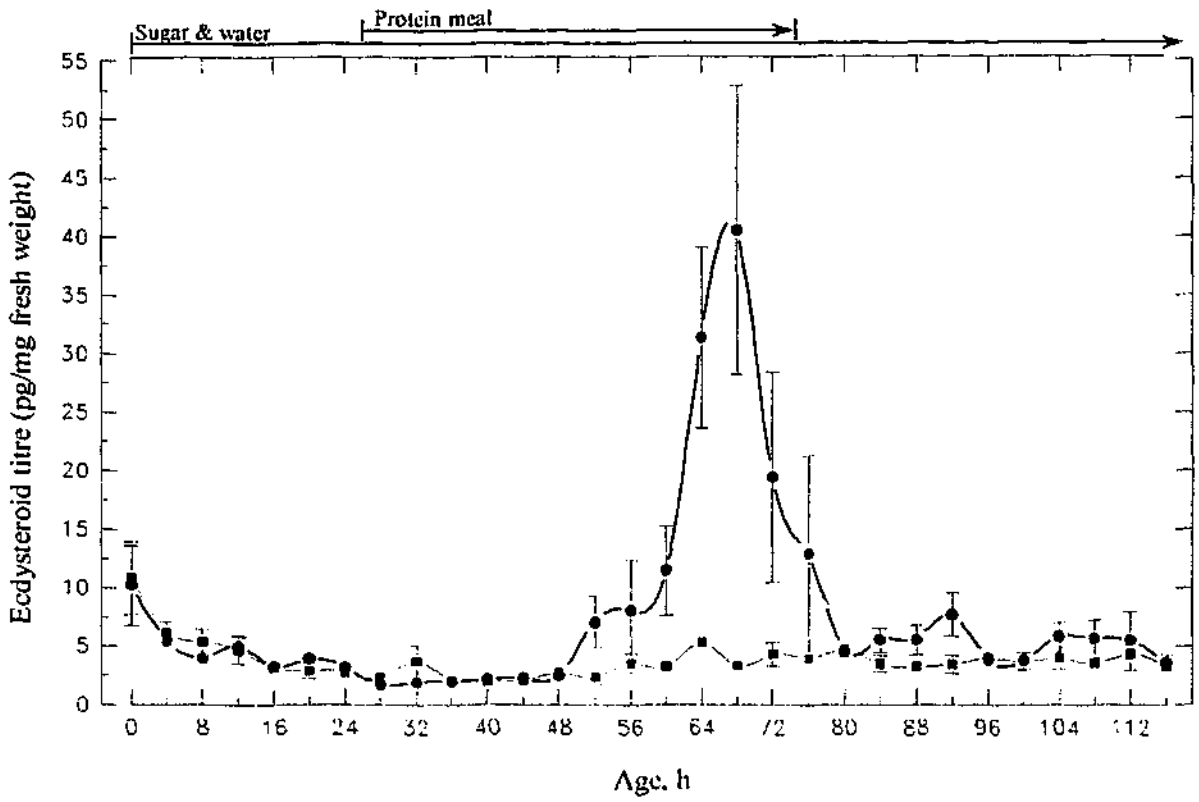


Fig. 47. A comparison of ecdysteroid titres measured by RIA between female (solid circles) and male (solid squares) adults of *Lucilia cuprina* reared at 27° C and fed with sugar, water and *ad libitum* lamb liver for 48 h starting on day 2. Each point represents the mean value ± SEM of 5 whole-body replicates, except where too small to show. Shaded areas indicate the scotophase.

Chapter 4

DISCUSSION

The results will first be discussed in terms of development, followed by endocrinological aspects will then be discussed in relation to specific developmental processes from the egg through to the adult stage.

4.1. Embryonic development

My results showed that the embryonic development of *L. cuprina* occurred over a relatively short period of time (10 - 11 h, at 27° C). Hourly observations in whole-mount specimens allowed detection and accurate timing of the developmental events occurring during embryogenesis (summarised in Table 2). My observations of *L. cuprina* embryos agree well with the general trend of embryonic development in the closely related species *L. sericata* (Davis, 1967). The faster embryogenesis in *L. cuprina* (10 - 11 h) in this study as compared with *L. sericata* (14 h; Davis, 1967), may, at least in part, be explained by the 2° C difference in breeding conditions (27° versus 25° C).

Because the early stages of cleavage division were completed in only 2 hours and could not be detected in whole-mounts, comparison with other studies can only be made in the second half of the stage, where organogenesis was clearly visible. The segmental arrangement of the embryonic body of *L. cuprina* observed 6 - 7 h after oviposition appears to be comparable to the segmental arrangement of muscles, which developed by 10 h following oviposition, in *L. sericata* at 25° C (Davis, 1967). In *L. cuprina* this event was followed by the appearance of the tracheal trunks (Fig. 9). This is in contrast with the results reported for *L. sericata* where the tracheal trunks formed approximately 1 h

before the segmental feature appeared. A possible explanation for this discrepancy is that the newly formed tracheal trunk could not be observed in *L. cuprina* whole-mount specimens. The timing of the appearance of the tracheal trunk is important as it is used as an indicator of the beginning of embryonic cuticle deposition. This has been clearly demonstrated by Hillman and Lesnik (1970) in embryos of *D. melanogaster* using a histological method and electron micrography, where the secretion of the cuticle took place at approximately the same developmental time throughout the organism. Evidence presented here (Fig. 9) shows that this is also the case in *L. cuprina*.

The subsequent developmental events (Fig. 10), *i.e.* the tanning of the tracheal trunks and the appearance of the gut, followed by sclerotisation of the posterior spiracles and mouth hooks, occurred in the same sequence in both *L. cuprina* and *L. sericata* (Davis, 1967). Furthermore, a similar sequence of events is found in late embryonic development of *N. bullata,* as reported by Wentworth and Roberts (1984). In this viviparous fly, in which the completion of embryonic development and hatching occur in the uterus, the appearance of the tracheal trunks and their tanning, as well as that of the posterior spiracles and mouth hooks characteristically occur in the same sequence. The data are thus in good agreement with these findings.

4.2. Ecdysteroids in the embryos

I have shown that in *L. cuprina* the ecdysteroid titre increases as a single peak during embryonic development (4 h after hatching, Fig. 12). A similar profile was reported in other dipteran species using the RIA method: in *N. bullata* (Wentworth and Roberts, 1984) and *D. melanogaster* (Maróy *et al.,* 1988). In *N.*

bullata, embryogenesis occurs in the uterus, and a maximal value of ecdysteroid titre was reached at 25% through embryogenesis. In *D. melanogaster*, where hatching occurs after oviposition as in *L. cuprina*, the ecdysteroid peak occurred approximately at 8 h of a 22 h embryogenesis at 25° C. This timing of 33% through the stage corresponds to that found here for *L. cuprina* and suggests that this ecdysteroid profile may be typical of all cyclorraphous dipterans.

Little is known, however, about the exact origin of ecdysteroids in *L. cuprina* embryos. Two possibilities may exist from studies of other species: ecdysteroids could come from *de novo* synthesis by the embryo itself and/or maternal origin. *De novo* synthesis would only be possible from tissues other than the prothoracic gland, such as oenocytes (Romer *et al.,* 1974) and integument (Cassier *et al.*, 1980), because the prothoracic gland in Diptera, a part of the retrocerebral complex, does not develop until at least the midpoint of embryonic development *i.e.* after the peak at 33%. This is evident in *L. sericata* (Davis, 1967) where the nervous system starts to develop at approximately 7 h within the 14 – 20 h embryogenesis. Furthermore, in *D. melanogaster,* morphological differentiation of neurones in the CNS occurs approximately at 9 – 10 h within a 22 h embryogenesis (Campos-Ortega and Hartenstein, 1985). This is followed by the formation of functional synapses which takes place approximately at the midpoint (11 – 13 h) of embryonic neurogenesis (Carlson and Hilgers, 1997). It is not until 16 h that the CNS is functionally competent *i.e.* prior to hatching (Goodman and Doe, 1993). It is thus unlikely that the peak of ecdysteroids at 4 h after hatching comes from *de novo* synthesis by the functional PG.

Although the source of embryonic ecdysteroids has not, as yet, been investigated in *L. cuprina,* evidence from other species indicates that a significant

contribution may come from a maternal source. In *Locusta migratoria,* Goltzené *et al.* (1978) convincingly demonstrated that the follicle cell epithelium of the ovaries is a source of ecdysteroids. This was also demonstrated in the cockroach *Nauphoeta cinerea* by Zhu *et al.* (1983); Zhu and Lanzrein (1984). The ecdysteroids produced by the follicle cell epithelium are then stored in a conjugated form in the yolk as a hormone reserve. Injections of labelled ecdysteroid precursor, [4-^{14}C]cholesterol, into adult females of *Schistocerca gregaria* before the start of ovarian ecdysteroid synthesis was found incorporated into ecdysteroids in the form of [^{14}C]ecdysteroid conjugates in the newly-laid eggs (Isaac and Rees, 1985). Conjugated ecdysteroids bound to yolk proteins were also found in *D. melanogaster* embryos (Bownes *et al.*, 1988).

There is evidence that during development, embryos hydrolyse conjugates into free ecdysteroids. This has been demonstrated by Isaac and Rees (1985) in *S. gregaria* that developing embryos injected with isolated [^{14}C]ecdysteroid conjugates contained free [^{14}C]ecdysteroids which were assumed to be the product of hydrolysis of [^{14}C]ecdysteroid conjugates. Furthermore, it was shown in *D. melanogaster* by Maróy *et al.* (1988) using RIA and HPLC, that the ecdysteroid composition changes during embryonic development from low polar conjugates at an early stage (6 h) to free ecdysone and 20-hydroxyecdysone during the peak at 8 h, and finally to ecdysteroids of mostly higher polarity at 12 h. Moreover, the A-2 antiserum used in the present research binds specifically to ecdysone, 20-hydroxyecdysone, inokosterone and ponasterone A (Bollenbacher, personal communication). Since the immunoreactivity of conjugated forms of ecdysteroids is much lower than that for the free forms (Lagueux *et al.*, 1981), and because the level of RIA-positive material was low during the early stage of embryonic

development, this suggests that few free ecdysteroids are present at first. The free ecdysteroid peak detected during embryogenesis in *L. cuprina* may hence be due to hydrolysis of maternal ecdysteroid conjugates.

Another important point to discuss is the possible function of the peak of free ecdysteroids during *L. cuprina* embryogenesis. My data show that the single peak is well correlated with the initiation of segmentation of the embryo followed by organogenesis. As mentioned earlier, ecdysone expresses its effects on development through its interaction with the ecdysteroid receptors. Significant levels of an ecdysone receptor LcEcR mRNA were detected in *L. cuprina* embryo (Hannan and Hill, 1997), suggesting a significant role of ecdysone during embryogenesis. Unfortunately, the correlation of this receptor to specific stages of embryogenesis has not as yet been demonstrated in *L. cuprina*. Studies of ecdysteroid nuclear receptors superfamily members have been carried out, however, in *D. melanogaster* (reviewed by Thummel, 1995). In this species, at least eight nuclear receptors have been identified as to carry out specific functions during embryonic development: knirp (kni) (Nauber *et al.,* 1988), knirp-related (knrl) (Oro *et al.,* 1988), embryonic gonad (egon) (Rothe *et al.,* 1989), tailless (tll) (Pignoni *et al.,* 1990), dHNF4 (Zhong *et al.,* 1993), seven-up (svp) (Mlodzik *et al.,* 1990), FTZ-F1 (Lavorgna *et al.,* 1991), and DHR39 (also called FTZ-F1β) (Ohno and Petkovich, 1992). Proper initial segmentation of *D. melanogaster* embryonic body requires ecdysteroid receptor members which are encoded by kni (Nauber *et al.,* 1988) and tll (Pignoni *et al.,* 1990) genes. The present study appears to support these findings, as the peak of ecdysteroid titre was followed by segmentation of the embryo (Table 2).

Ecdysteroids may also have a significant role in organogenesis. This is evidenced by the fact that organogenesis of the midgut and Malpighian tubules in *Drosophila* requires the expression of dHNF4 (Zhong *et al.*, 1993). Furthermore, a complex early ecdysone-inducible gene, E75A, which was originally known to be active during *D. melanogaster* metamorphosis, was also found to be required in embryonic gut morphogenesis (Bilder and Scott, 1995). In addition, the tll gene is also required for the development of the gut, brain, and Malpighian tubules (Pignoni *et al.*, 1990). Evidence of organogenesis in the present study is indicated by cuticulogenesis, which involves the formation of the tracheal trunks and the tanning of posterior spiracles, as well as the appearance of the gut. These three events occurred 4 to 5 h following the ecdysteroid peak (Table 2), suggesting a role of ecdysteroids. Furthermore, it was demonstrated in *D. melanogaster* that the formation of embryonic cuticle occurred synchronously throughout the organism between 12 and 16 h of embryonic development (Hillman and Lesnik, 1970). Later, Maróy *et al.* (1988) measured the ecdysteroid titre in *D. melanogaster* embryos and found that the ecdysteroid peak occurred at 8 h of embryonic development, or 4 - 8 h before cuticulogenesis. The above data suggest that the free ecdysteroid pulse in *L. cuprina* embryos may regulate cuticulogenesis and gut formation, and probably organogenesis in general.

In conclusion, the single peak of free ecdysteroid during embryogenesis apparently performs multiple roles including proper initial segmentation of the embryo followed by organogenesis, through its interaction with its nuclear receptors, presumably expressed differentially at specific stages throughout development. This mechanism does not, however, exclude the possibility that other hormones reported to be present in embryos such as JH (in *Hyalophora*

cecropia, Gilbert and Schneiderman, 1961; in *Oncopeltus fasciatus*, Dorn, 1975, and Bergot, 1981; in *Locusta migratoria*, Roussel and Aubry, 1981) and PTTHs (in *Manduca sexta*, Dorn *et al.*, 1987) may also be present in *Lucilia* embryos. The possible interrelationships between ecdysteroids and other hormones regulating embryogenesis of *L. cuprina* still remain to be investigated.

4.3. Larval development

Larval development in *L. cuprina* is punctuated by two moults: the first 18 h after hatching and the second at 34 h. In the viviparous flesh flies, *Tricholioproctia impatiens* larval moults occurred 28 and 52 h following larviposition at 25° C (Roberts, 1976), while in *N. bullata* (Wentworth and Roberts, 1981) moults occurred at 24 and 44 h at the same temperature. This indicates that the length of each larval instar may vary according to species, independently of rearing temperature.

In *L. cuprina*, as in all cyclorraphous dipterans, larval development thus passes through three instars, which were conveniently identified by the mouth hooks. As the cuticle, including the cephalopharyngeal apparatus, is shed at the end of each instar, a new and larger pair of mouth hooks develops, presenting a distinct morphology for each instar (summarised in Table 3). The shape of first instar mouth hooks in *L. cuprina* was found to differ dramatically from that of *L. sericata* studied by Menees (1962). In *L. cuprina*, first instar mouth hooks curve dorsally with relatively small hooks near their anterior ends; whereas in *L. sericata* the mouth hooks curve ventrally with their distal ends appearing relatively larger. In the second and third instar, however, the morphology of mouth hooks appeared very similar in both species.

Interestingly, the mouth-hook length increased progressively until about half way through the first instar and through two thirds of the second instar, and decreased again later within each instar. In the third instar, the mouth hook size appeared at first to increase continuously and then remained relatively constant (Table 3). The increase in size following hatching or a moult may be due to cuticular deposition, and the subsequent decrease in size toward the end of the first and second instars could be caused by partial lysis of the mouth hooks. It should be noted that the above phenomenon was not found in *T. impatiens* (Roberts, 1976) or *N. bullata* (Wentworth and Roberts, 1981) who measured only the dimension of the sclerotised mandibular hook. In this study, I measured procuticular processes which are deposited and resorbed during the instar. Cuticular deposition and resorption are well documented in *Locusta sp.* (Neville, 1975). However, the precise mechanisms involved are still unknown. The recent report of the presence of gelatinolytic activity in the haemolymph of feeding *N. bullata* larvae and its disappearance in the wandering stage (Bylemans *et al.*, 1997) may provide an insight in these mechanisms, as these enzymes may be involved in remodelling tissues, including the cuticle that stretches enormously during larval growth.

The behaviour of larvae at the end of the third instar is noteworthy. Results show that the last larval instar exodus from the liver was completed over three consecutive nights (Fig. 19), and was almost totally confined to the scotophase, suggesting that photoperiod may be an environmental cue for this behaviour. Smith and collaborators (1981) demonstrated, however, that *L. cuprina* larvae maintained under a light/dark cycle until the beginning of exodus,

and then shifted to constant darkness showed circadian peaks of exodus, suggesting the involvement of an endogenous rhythm.

Furthermore, time of oviposition also appears to influence larval exodus from food. Results (Figs. 18 and 19) from three replicate groups originating from eggs laid at different times throughout a 24 h period show that the later the time of oviposition, the later the start of the exodus peak. The data presented here are different from those reported by Smith *et al.* (1981) for a group corresponding to my replicate A, in that the largest exodus of larvae (approximately 70 %) was reached (Fig. 18, A) in the late first night rather than the early second night as reported by Smith *et al.* (1981). A possible explanation may be a difference in diet between the two studies: I used pure liver while Smith and colleagues used a mixture of liver and cotton linters. More nutrients being available for larval development in this research may account for a faster growth of larvae. This suggests that the exodus of *L. cuprina* may be controlled by a similar mechanism as that found in the tobacco hornworm *Manduca sexta* (Nijhout and Williams, 1974; Truman and Riddiford, 1974; Nijhout, 1975) where the larvae need to have reached a certain weight to be responsive to photoperiod before leaving the food.

Finally, weight monitoring throughout larval development (Fig. 20) shows that larval weight in *L. cuprina* increased dramatically during the first and second instars and continued during the early third instar. The loss of weight found during the post-feeding larval stage is due to the emptying of the crop before pupariation. Similar trends have been noted in the blowfly *C. stygia* (Kinnear *et al.*, 1968), and the flesh flies *Tricholioproctia impatiens* (Roberts, 1976) and *N. bullata* (Wentworth and Roberts, 1981).

4.4. Ecdysteroids in the larvae

Three distinct and large peaks of whole-body ecdysteroid titres were observed throughout the larval stages of *L. cuprina*. The first two peaks occurred at 8 h and 26 h prior to the first and second larval/larval moults at 18 and at 34 h respectively, and the third one towards the end of the third instar (summarised in Figs 21 to 24). The occurrence of three ecdysteroid peaks was also reported for *N. bullata* reared at 25° C under LD 16 : 8 photoperiodic conditions (Wentworth *et al.*, 1981). In the latter species the first and second peaks occurred at 14 and 30 h respectively, prior to larval/larval moults at 24 and 44 h respectively, and the third peak occurred at 131 h by the end of the third instar. The absence of a surge in ecdysteroid levels during the leaving of the food (Fig. 23) is in good agreement with ultrastructural examinations of the prothoracic gland in *L. cuprina* by Meurant and Sernia (1993). These authors found that the infolding of the prothiracic gland cell membranes was not prominent during the commencement of post-feeding stage indicating an inactive state of the gland. A similar profile was also reported by Lee (1992) in the blowfly *P. regina* reared at 25° C under LD 16 : 8 photoperiodic conditions. Thus, my data support these findings.

Furthermore, my RIA results during late third instar of *L. cuprina* are in good agreement with other dipteran *in vitro* measurements of ecdysteroid synthesis by the prothoracic glands in *N. bullata* by Roberts (1976), who clearly demonstrated in this species that the synthetic activity of the ring gland increases at the end of wandering stage and peaks close to pupariation (Roberts, 1976). Similar results were also found in *D. melanogaster* by Dai and Gilbert (1991) using the same technique in conjunction with ultrastructural examination of the gland. Thus, the high levels of ecdysteroids in whole bodies during late third

instar of *L. cuprina* appear to result directly from the high activity of the prothoracic gland.

The possible mechanism of control of ecdysteroids over the moulting process in *L. cuprina* appears to be similar for the first two larval instars since the first two ecdysteroid peaks during these stages were well correlated with the first and second larval-larval moults, and the ecdysteroid titre profiles followed a similar pattern. Each of these two peaks was followed by a sharp decrease in ecdysteroid levels. This phenomenon during the feeding stage of larval *L. cuprina* appears similar to patterns observed in *D. melanogaster* (Talbot *et al.*, 1993) and *M. sexta* (Thummel, 1995). The rise and fall of titre of ecdysteroids control a period of intense gene expression (Talbot *et al.*, 1993) which specifies the phenotype of the next developmental stage (Thummel, 1995). The increasing titre of ecdysteroids turns off expression of genes associated with intermoult activities of the epidermal cells, and the following decline of ecdysteroid level turns on the expression of the "moult gene" (Riddiford and Hiruma, 1990). More precisely, at each peak, ecdysteroids induce expression of the EcR and ETH gene in Inka cells and receptors for PETH and ETH in the CNS. Subsequent low level of ecdysteroid titre at the end of each moult is required for the release of ETH, which initiates the ecdysis behavioural sequence: PETH triggers pre-ecdysis I, while ETH activates pre-ecdysis II and ecdysis (Zitnan *et al.*, 1999). My data clearly showed that ecdysis occurred during the decline of ecdysteroid levels in both the first and second instars (Fig. 24), thereby supporting the above evidence.

In contrast to the first two peaks, the data indicate that the large prominent ecdysteroid level at the end of the third instar of *L. cuprina* is almost certainly related to the process of pupariation. A similar peak in moulting hormone activity

prior to pupariation was found in a previous study in this species using the *Musca* bioassay (Barritt and Birt, 1970). This was also reported in other dipteran species using RIA: namely *N. bullata* (Wentworth *et al.*, 1981) and *C. capitata* (Vafopoulou *et al.*, 1993). Our knowledge of the underlying processes of pupariation, as a response to the increase of ecdysteroid levels during the late larval stage of development in *L. cuprina,* is still far from complete. Nevertheless, during this stage, significant levels of LcEcR mRNA resembling the B1 isoform of DmEcR have been detected in *L. cuprina* (Hannan and Hill, 1997). EcR is induced directly by ecdysone, providing an auto-regulatory loop that increases the level of receptor protein (Talbot *et al.*, 1993). In *D. melanogaster,* the late larval response to ecdysone is characterised by a precise sequential activation of members of the superfamily of nuclear receptors. The EcR-USP binds ecdysone and an ecdysone response element (EcRE) in the DNA, activating a downstream promoter to perform transcriptional activity. Many of these genes are localised in the polytene chromosome puffs of the salivary gland (Huet *et al.*, 1995). In *C. capitata,* the major changes in puffing activity occur around jumping, a characteristic behaviour occurring before puparium formation. The puffing activity is regulated by ecdysteroids (Gariou-Papalexiou *et al.*, 1999). Thus, the high level of ecdysone observed in the present study during the late third larval stage in *L. cuprina* may be responsible for expression of LcEcR-B1, which in turn regulates transcriptional activities in the target tissues leading to pupariation. This remains to be further investigated in *L. cuprina*.

In addition, I observed that tanning and sclerotisation occurred within one hour of the puparium formation in *L. cuprina*. The ecdysteroid peak at this stage has also been correlated to a peak of dopa-decarboxylase activity in *C.*

erythrocephala (Shaaya and Sekeris, 1965). Furthermore, Clark *et al.* (1986) demonstrated that 20-hydroxyecdysone induces the activity of dopa-decarboxylase mRNA in larval epidermis of *D. melanogaster*. The activation of dopa-decarboxylase leads to the tanning process of the puparium (Sekeris and Fragoulis, 1985). While high levels of ecdysteroids appear to be necessary for the tanning process in *L. cuprina,* there is evidence that ecdysteroids may not be the only requirement for initiating pupariation, sclerotisation and tanning. Once a post-feeding third instar larva is developmentally committed to metamorphosis after being exposed to high levels of ecdysteroids together with low levels of JH, the subsequent pupariation behaviour and tanning appear to be regulated by a neural mechanism. This was exemplified in the tsetse fly *Glossina morsitans* by Zdarek and Denlinger (1992). They found that a sensory input from the extreme posterior part of the body inhibits pupariation and tanning. In order for pupariation to proceed elimination of this sensory signal is required. However, this still needs to be verified in *L. cuprina.*

It is noteworthy that each ecdysteroid peak found throughout the larval stages of *L. cuprina* occurred during the scotophase (Figs. 21 - 24). A similar trend is found in last instar larvae of *Locusta migratoria,* where the activity of the prothoracic gland fluctuates slightly with light-dark cycles, but reaches a maximum at the end to the dark period (Roussel, 1992). Evidence from *N. bullata* presented by Roberts (1984) may provide an explanation for the occurrence of ecdysteroid peaks during the scotophase. This author showed *in vitro* that the prothoracic gland activity is directly correlated to PTTH release and is high only for 2 h approximately midway through the scotophase. PTTH release was also reported to occur for a period of about 3.5 h during the scotophase in lepidopteran

Manduca sexta (Truman and Riddiford, 1974). More recently, the existence of a daily rhythm of PTTH release was reported in *Rhodnius prolixus*: PTTH releases follow a circadian clock regulated by the brain which is entrained by extraretinal photoreception (Vafopoulou and Steel, 1996). Thus, the 12 : 12 light / dark cycle used in the present study may have entrained the circadian clock of PTTH releases during larval stage of *L. cuprina*, thereby causing ecdysteroid synthesis during the scotophase. Further investigations are needed, however, to verify this phenomenon. It would be interesting to follow the profile of PTTH release in *L. cuprina* to investigate the exact role of photoperiod in these events.

4.5. Prepupal development

The data show that immediately following the wandering period, *L. cuprina* post-feeding larvae became immobile. Permanent cessation of movement is followed by retraction of the three anterior larval segments and further body shortening to form an ovoid shape. This behaviour is in agreement with that described in *N. bullata* (Zdarek and Fraenkel, 1972; Zdarek and Friedman, 1986). Furthermore, these authors demonstrated that the body shortening is due to a longitudinal muscle contraction and shrinkage in the cuticle. These events are followed by a phenolic stabilisation of the contracted cuticle and a rapid water loss in the cuticle, which together with tanning, result in complete loss of its elasticity. This condition leads to an increase in the internal body pressure (Zdarek *et al.*, 1979) which may have an impact on the ultra-structural rearrangement of protein-chitin micelles in the cuticle, resulting in a reduction of cuticular surface area (Fraenkel and Ruddall, 1940). The high pressure resulting

from these processes causes the ultimate smoothening of the surface of the white prepupal body (Zdarek *et al.*, 1979). This event was also observed in *L. cuprina.*

Furthermore, the data show clearly that when the larvae become rounded in shape and immobile (pupariation), the entire epidermis is still attached to the puparium (Figs. 25, A, B, and C). It is not until approximately 8 h later that the larval/pupal apolysis occurred (Fig. 26, A and B). This is in contrast with Barritt and Birt (1971), who reported that the larval/pupal apolysis occurred simultaneously with the larva becoming rounded and immobile, referring to this stage as the white pupa. This discrepancy could be explained by the fact that the process of pupariation was accelerated at 30° C in such a way that these authors measuring at 4 h intervals, may have missed the prepupal stage. During the period between pupariation and larval/pupal apolysis, the animals are in the stage of prepupa, a transitional stage between larval and pupal stage which is peculiar to most cyclorraphous flies. My results clearly show that the use of the term white pupa to refer to the untanned puparium is erroneous, and the term white prepupa is more appropriate, as suggested earlier by Fraenkel and Bhaskaran (1973).

Almost immediately after the cuticular smoothening of the white-prepupal *L. cuprina,* tanning and sclerotisation begin and continue until the cuticle becomes dark brown in colour. Cuticular sclerotisation involves the process of transfer of an ortho-diphenol compound such as N-acetyldopamine (NADA) or N-β-alanyldopamine (NBAD) into the existing third instar larval cuticle (Andersen, 1990). The level of NADA in the drosophilid fly *Chymomyza costata* was found to be elevated during prepupal tanning (Kostal *et al.*, 1998). The sclerotising compounds (diphenols) which are secreted by the epidermal cells need to be enzymatically activated before reacting with the cuticular macromolecules and

form inter-protein cross-links. The activating enzyme, which is generically called phenoloxidase, is located in the epicuticle (Andersen, 1990). In *L. cuprina* two types of phenoloxidases (tyrosinases and laccases) are found in the epicuticle of larvae about to pupariate (Binnington and Barrett, 1988). Thus the dark brown colour of the puparium of *L. cuprina* may be caused by β-alanine pigmentation.

Finally, the loss of the larval muscle striations observed in *L. cuprina* after pupariation (Fig. 25, C) is also in agreement with the previous observations by Barritt and Birt (1971). This loss indicates the beginning of larval muscle degeneration and reconstitution into the imaginal musculature. This may coincide with a sharp increase of total acid proteolytic activity, as observed in the medfly *Ceratitis capitata* from 0 to 44 h after puparium formation (Rabossi *et al.*, 2000). Based on the activation of lysosomal markers, such as acid phosphatase and beta-glycosidases, these authors reported that this proteolytic activity is lysosomal. They also suggested, based on the results of endopeptidase inhibitor assays, that members of the aspartic and cystein proteinase families are responsible for the degradation of larval tissues in dipterans. This remains, however, to be confirmed in *L. cuprina*.

4.6. Ecdysteroids in the prepupae

During the prepupal stage of *L. cuprina*, an ecdysteroid peak occurred 4 h after pupariation, which was followed by a sharp decline of ecdysteroid level. This is similar to the single surge of ecdysteroid reported shortly after pupariation in *P. regina* (Lee, 1992), *N. bullata* (Wentworth *et al.*, 1981), *Boettcherica peregrina* (Moribayashi *et al.*, 1992) and *C. capitata* (Vafopoulou *et al.*, 1993).

The probable source of ecdysteroid during prepupal stage of *L. cuprina* is the prothoracic gland. Although PG activity has not directly been measured in *L. cuprina*, the PG was found to be biosynthetically active during the early prepupal stage of other dipterans, as demonstrated *in vitro* in *N. bullata* (Roberts *et al.*, 1984) and in *D. melanogaster* (Redfern and Bownes, 1984). Two hours later, however, the biosynthetic activity begins to decrease to a barely detectable level 21 h (in *N. bullata*) and 30 h (in *D. melanogaster*) after pupariation. The data in the present study appear to be in agreement with these findings, as after a short period of peak, ecdysteroid levels decline dramatically in *L. cuprina*. This may be partly due to the beginning of degeneration of the PG cells as observed in *D. melanogaster* by Dai and Gilbert (1991). In this species, the ultrastructure of the prothoracic gland changed after puparium formation. Inactivity was indeed indicated by the reduction of smooth endoplasmic reticulum and mitochondria, absence intercellular channels, and cytoplasmic fragmentation. This was accompanied by a dramatic decrease in the capacity of ecdysteroid synthesis. Although ultrastructural changes were not studied in *L. cuprina* prepupae, these may nonetheless have occurred.

The probable function of prepupal ecdysteroid peak is most likely to initiate the larval/pupal moult, since apolysis started 8 h after pupariation and completed within the next 4 h (Table 4). Interestingly, the ecdysteroid surge during this stage results in a different moult *i.e.* larval/pupal (instead of larval/larval). Early in every moult, there is a critical commitment period during which the presence/absence of active JH determines a switch in gene expression in response to the increasing ecdysteroid levels (Riddiford, 1994). This commitment period is followed by a pre-differentiative period up to the peak of

ecdysteroid level. During the decline of ecdysteroid level a differentiative period occurs. It has been shown in *D. melanogaster* (Talbot, 1993) and in *M. sexta* (Jindra *et al.*, 1996) that in the presence of JH, an isoform of ecdysone receptor, the EcR-B1, is predominant during the critical commitment and pre-differentiative period of the larval/larval moult. This is followed by the appearance of another isoform, EcR-A, which is predominant at the onset of cuticle deposition. In contrast, in the absence of JH during the onset of a metamorphic (either the pupal or the adult) moult, EcR-A appears earlier during the later part of the commitment phase and persists through the pre-differentiative phase until the onset of cuticle deposition. This suggests that different isoforms, or isoform combinations of ecdysone receptors result in different responses of tissues to ecdysone. In *L. cuprina*, *in vitro* rates of JH_3 biosynthesis reach a peak during the PFL stage and decline sharply during the prepupal stage (Sutherland, 1997). My data indicate that the prepupal ecdysteroid peak is followed by larval/pupal apolysis and pupal cuticle deposition 8 h after pupariation (Fig. 26, B). Thus, the present data support the findings that the prepupal peak of ecdysteroid, accompanied by a low level of JH, regulates the expression of ecdysone receptor isoforms leading to the commitment to a larval/pupal moult.

Furthermore, the data indicate that during the decline of prepupal ecdysteroid levels, larval muscle degeneration starts in *L. cuprina* (Fig. 26, B). Data from *D. melanogaster* (Talbot *et al.*, 1993) indicate that the EcR-B1 isoform is expressed primarily in larval tissues including the muscle that are fated to degenerate and die during metamorphosis. The death of these tissues is dependent on a decrease in ecdysone level (Robinow *et al.*, 1993). My data show that during the decline of ecdysone level (8 h after pupariation), the muscle had lost its

striation indicating the start of larval muscle degeneration (Fig. 26, B). Thus, the data in *L. cuprina* support the finding that after being exposed to high levels of ecdysone, larval muscle expresses EcR-B1 isoform, and the following decline of ecdysone levels leads to larval tissue degeneration.

4.7. Pupal and pharate-adult development

The histological data indicate that in *L. cuprina* the larval/pupal moult occurred soon after the completion of sclerotisation and tanning of the puparium (Fig. 26, A). This moult, as indicated by the separation of the epidermis from the puparium, started from the anterior part of the body and was accompanied by the deposition of the pupal cuticle (Fig. 26, B). At this stage the epidermal cells were thicker than those in the prepupal stage, and their nuclei rounded rather than elongated. This may be related to nucleic activity in cuticle synthesis (Fig. 26, B). These events in *L. cuprina* agree well with those observed in the equivalent stage in *N. bullata* by Fraenkel and Bhaskaran (1973).

Furthermore, the data show that the appearance of the division of the pupal body into a distinct thorax and abdomen in *L. cuprina* occurred approximately by 24 h after pupariation. In this cryptocephalic pupa, the abdomen occupies about three quarters of the pupal body, which is due to the presence of the large gas bubble in the intestine. Barritt and Birt (1971) observed that the cryptocephalic pupa occurred within 10 h of pupariation at 30° C, thus occurring earlier than in this study, probably owing to higher rearing temperature. The appearance of the large gas bubble filling the midgut was also observed in the same stage in *N. bullata* by Zdarek and Friedman (1986). These authors found that the gas bubble was externally visible in a depupariated whole-mount specimen at some 15 - 20 h

after pupariation. Moreover, the authors observed that the leg appendages were in the form of flat tubes attached to the latero-ventral side of the thorax, while the wings were latero-dorsal. These thoracic appendages could not be shown in this study as the data presented here used sagittal sections rather than whole-mount material.

The evagination of the head in *L. cuprina* pupa was observed 28 h post-pupariation. During this process, the brain migrated to the cranial region, while the gas bubble disappeared from the gut. Again head evagination was observed earlier (16 h post-pupariation) under higher temperature condition by Barrit and Birt (1971). The dynamic morphogenetic events of *L. cuprina* metamorphosis agree well with those observed in histological sections of *N. bullata* (Fraenkel and Bhaskaran, 1973). The disappearance of the gas bubble, presumably after abdominal contraction, resulted in the deflation of the abdomen observed in the present study (Fig. 29). This event has also been clearly shown in *N. bullata* by Zdarek and Friedman (1986) using pupae removed from the puparium. Using a barograph these authors found that a series of peristaltic contractions of the abdominal muscles increase the internal pressure of the haemolymph, which with the aid of the large gas bubble result in the inflation of the thoracic appendages and the evagination of the head to form the phanerocephalic pupa. These events were followed by the migration of the imaginal organs such as the brain, tracheae, fat body to the newly formed imaginal structures.

Finally, the data indicate that the pupal/adult apolysis had occurred by approximately 40 h post-pupariation (Fig. 29). Following the pupal/adult apolysis the definitive adult morphology has been achieved and the animal is in the stage

of the pharate adult. Barritt and Birt (1971) observed this stage approximately 32 h after pupariation. A similar feature was also observed in histological sections in *N. bullata* by 96 h post-pupariation (Fraenkel and Bhaskaran, 1973) and in whole-mounts of the same species (Zdarek and Friedman, 1986).

4.8. Ecdysteroids in the pupae and pharate adults

By the time the pupa had formed, the ecdysteroid levels had decreased and then increased again 36 h after pupariation, which was later followed by pupal/adult moult, marking the beginning of pharate adult stage. During the pharate adult stage, the first ecdysteroid peak occurred between 60 and 68 h in females (Fig. 33), and at 56 h in males (Fig. 34). The second pharate adult peak of ecdysteroids occurred at 92 h after pupariation in both males and females. The same profile was also observed in *N. bullata* (Wentworth *et al.*, 1981).

Little is known about the exact sources of free ecdysteroids during pupal and pharate adult stages of development. During the pupal stage, a possible source of ecdysteroid is most likely the PG, since data from *D. melanogaster* (Dai and Gilbert, 1991) and *N. bullata* (Roberts *et al.*, 1984) indicate that the PG is still biosynthetically functioning. During the pharate adult stage, on the other hand, the biosynthetic activity of the PG is barely detectable, and tissues other than the PG are likely sources of ecdysteroids. Alternative sources of ecdysteroids may be either ecdysiosynthetic or storage tissues that release stored conjugated forms of ecdysteroids (Bownes, 1990). In addition, because a pupa is a closed system and uses its reserves for living, like an egg does, it is possible that both systems share the same way of utilising hormone reserves (Delbecque *et al.*, 1990). Further investigation is however needed to verify these propositions in *L. cuprina*.

The apparent function of the increased ecdysteroid levels at the end of the pupal stage of *L. cuprina* is likely to initiate pupal/adult apolysis, which occurred at 40 h (Fig. 30). The first pharate adult peak of ecdysteroid levels, on the other hand, may be related to various developmental events. In *D. melanogaster*, expression of EcR-A isoform reaches a peak during the same stage in the imaginal discs including the wing discs, the leg discs, the haltere discs, the labial disc, and the eye-antennal disc (Talbot *et al.*, 1993). In contrast to EcR-B1 expressed in larval tissues, EcR-A is expressed in developing adult structures and tissues. Expression of EcR-A during this period indicates that adult organ differentiation is taking place at this time, as a response to the increase of ecdysteroid levels. It is evident that in *L. cuprina*, a variety of developmental events pertaining to the morphogenesis of thoracic legs and flight muscles, eye pigmentation, leg segmentation with setae, hairs, claws and the pulvillus occurred following pupal/adult moult (Barritt and Birt, 1971; Table 4). Thus the first pharate adult peak in *L. cuprina* may regulate developmental events leading to adult organ differentiation.

In addition, it is noteworthy that the first pharate adult peak of ecdysteroids occurred earlier in males than in females. This difference was also reported using HPLC and RIA methods in the mosquito *Aedes aegypti* (Whisenton *et al.*, 1989) and the hevea tussock moth *Orgyia postica* (Gu *et al.*, 1992). These authors suggested that this difference between male and female ecdysteroid profiles is responsible for the sexual dimorphism of developmental rates. In *L. cuprina*, it was shown that the majority of adult males eclose one day earlier than the females (Vogt *et al.*, 1974). However, this needs further investigations in *L. cuprina*. It would be interesting, for example, to correlate

ecdysteroid titres with microscopic developmental events in the reproductive organs in both sexes.

In contrast to the first pharate adult ecdysteroid peak, the second peak occurred at the same time in female and male pharate adults of *L. cuprina*. This peak is associated with continued differentiation of all imaginal structures (Barritt and Birt, 1971), deposition of adult endocuticle and pre-eclosion tanning (Whitten, 1969). A similar peak was also observed in *D. melanogaster* (Bainbridge and Bownes, 1988). This peak was associated with the formation of head and thoracic bristles and the darkening of the wings.

4.9. Adult female development

My data indicate that a liver meal significantly increases body weight of liver-fed females (Fig. 35). Thereafter, the body weight remained relatively constant at least until the end of the first reproductive cycle. This appears to be a general weight profile in flies as similar situations were also reported in other blowflies such as *C. vicina, L. caesar,* and *P. terrae-novae* (Briers and De Loof, 1983) and in the fleshfly *N. bullata* (Wentworth, 1982).

The irreversible increase in whole-body weight before the onset of vitellogenesis (up to 48 h after eclosion) may reflect the build-up of reserve materials (*e.g.* lipids, proteins and glycogen) in the fat body, which is a primary site of storage and synthesis of molecules (Hagedorn *et al.*, 1973; Thomsen and Thomsen, 1974). The increase of reserve material after feeding may result from digestion of food as observed in *A. aegypti* (Felix *et al.*, 1991). Protein is digested with trypsin-like enzyme released in two phases. The first phase of trypsin synthesis is induced by soluble proteins of various molecular weights from the

diet which initiate formation of mRNA. The second phase of trypsin synthesis is a result of the translation of newly formed mRNA and is induced by soluble peptides with molecular masses of 1 – 10 kDa in the gut. The amount and type of protein consumed resulting in the transcriptional up-regulation of the late trypsin gene, determine the levels of late trypsin (Noriega *et al.*, 1994). Protein digestion provides amino acids for the synthesis of yolk proteins, *i.e.* vitellogenin, and resulting in ovarian maturation (Adams, 1999). As vitellogenin is synthesised in the fat body and secreted into the haemolymph (Hagedorn and Judson, 1972), this may cause an increase in haemolymph volume after protein feeding as observed in the blowfly *P. regina* by Yin *et al.* (1990), and may contribute to the increase of the whole-body weight.

Oogenesis in *L. cuprina* commenced approximately 21 h following protein feeding and was followed by yolk deposition, resulting ultimately in egg maturation. During this period, the mean weight of the female reproductive system progressively increased and reached a steady state near the end of the first reproductive cycle (Fig. 36). In contrast, the mean weight of the female bodies from which the reproductive system have been removed, decreased significantly during this period (Fig. 37). The simultaneous increase of the reproductive system weight and decrease of the carcass weight, which contained most of the fat body, can be explained by the fact that vitellogenin, synthesised in and released from the fat body, is transferred to the oöcytes and stored in yolk granules, as observed in *D. melanogaster* (Bownes *et al.*, 1993). The mechanism by which vitellogenin is transferred to the oöcytes may be the same as that observed in *A. aegypti* by Snigirevskaya *et al.* (1997): *i.e.* vitellogenin passes through the interfollicular spaces, binds with receptors on the oöcyte plasma membrane, and

enters the oöcytes by receptor-mediated endocytosis. It is evident in *A. aegypti,* that vitellogenin receptor protein is found primarily in the oöcyte cortex (Dhadialla *et al.,* 1992) and increases dramatically 8 – 24 h after the blood meal (Sappington *et al.,* 1995). Thus, the synchronous weight increase of the reproductive system and decrease of the carcas in *L. cuprina* may reflect the process of vitellogenin transfer from the fat body the oöcytes.

In contrast to liver-fed females, the mean body weight of non-liver fed females did not vary significantly over time, except during the first 8 h following adult emergence. The decrease in weight of whole-body adult females of *L. cuprina* during this period may be due to loss of water by transpiration through the cuticle and elimination of fluids through the anus, as observed in *N. bullata* by Cottrell (1962). These fluids presumably contain metabolic products stored in the intestine during metamorphosis (Schwartz *et al.,* 1989). The data in *L. cuprina* females support these findings. Thereafter, the mean body weight remained constant. This may reflect the fact that non-liver fed females lacked materials stored in their fat bodies. It was indeed found that non-liver fed females of *L. cuprina* had only a thin translucent sheet of fat body (Williams *et al.,* 1977). In addition, previous observations of non-protein fed females showed that ovarian development did not progress any further than differentiating one follicle (Clift and McDonald, 1973). Being an anautogenous strain, *L. cuprina* females need a protein diet to mature their ovaries (Williams *et al.,* 1979). This has also been reported in *M. domestica* by Adams and Gerst (1991) who found that flies fed only on sucrose did not mature ovaries.

In conclusion, the protein meal in *L. cuprina* appears to provide female flies with materials necessary for vitellogenin biosynthesis in the fat body.

Vitellogenin is then released into the haemolymph and transferred to and stored in the oöcyte as reserve proteins required for embryogenesis. This process is reflected by the dramatic increase of whole body weight following a protein meal.

4.10. Ecdysteroids in adult females

At emergence, ecdysteroids were detected (approximately 10 pg/mg fresh weight), and this was followed by a continuous decrease until barely detectable levels. Coinciding with the formation of the secondary ovarian follicle and the start of vitellogenesis in *L. cuprina* (Table 5), the whole-body ecdysteroid titre increased rapidly and peaked at approximately 68 h after emergence; this was followed by a continuous ecdysteroid decrease until the end of the first ovarian cycle (96 h). A similar pattern was also found in whole-body of *N. bullata* (Wentworth and Roberts, 1984), and *Culex pipiens* (Baldridge and Feyereisen, 1986). The data showed that the ecdysteroid profile of the reproductive system followed the same pattern as that of the whole-body with a peak at the same time, but approximately three times higher. Furthermore, the level of the ecdysteroids in the female carcass (from which the reproductive system had been removed) was low, suggesting that almost all ecdysteroids in *L. cuprina* adult females are located in the reproductive system. These data are in good agreement with the finding of Clissold *et al.* (1993), who reported a peak of ecdysteroids at 68 h post-eclosion in ovaries of blood-fed females of *L. cuprina*.

Although the possible sources of ecdysteroids at emergence and during early adult life have not been investigated as yet in *L. cuprina,* data in *D. melanogaster* show that approximately half of ecdysteroids detected at emergence are highly polar metabolites and stored during metamorphosis in the intestine

(Schwartz *et al.*, 1989). It is indeed not until adult eclosion that these metabolic products are excreted, since no means for excretion are available during metamorphosis. The present study in *L. cuprina* supports the finding that high levels of ecdysteroids in newly eclosed flies was followed by a sharp decrease in titres, after the release of the meconium. Several adult tissues may, however, be sources of ecdysteroids in newly eclosed flies. It has been demonstrated in *D. melanogaster* by Bownes *et al.* (1984) that the head and thorax, abdominal body wall, gut and Malpighian tubules, all contain and produce ecdysteroids that most likely contribute to the total whole-body ecdysteroid titre in adults. However, further investigations are needed to elucidate the exact sources of ecdysteroids at emergence and during early adult life in *L. cuprina*.

The possible sources of ecdysteroids detected later in adult female development, *i.e.* during the ovarian cycle in *L. cuprina* are most likely the ovaries. It has been demonstrated by Rubenstein *et al.* (1982) and Schwartz *et al.* (1985) that the ovaries of *D. melanogaster* are able to produce ecdysteroids when incubated *in vitro*. The site of ecdysteroid synthesis is more specifically the ovarian follicle cells (in *Locusta migratoria,* Goltzené *et al.*, 1978). Hence, the single major ecdysteroid peak in *L. cuprina* females during the reproductive cycle presumably results from ecdysteroid synthesis activity in the ovaries. *In vitro* assay of ovarian ecdysteroid biosynthetic activity would be useful to confirm this in *L. cuprina*.

The ecdysteroids contained in the carcass of adult females of *L. cuprina*, on the other hand, may have two possible origins. Firstly, these ecdysteroids could be a part of metabolic products which are in the process of elimination via the gut and Malpighian tubules (Isaac and Slinger, 1989) and/or some of the

ovarian ecdysteroids that may be released into the haemolymph, as shown in the ovoviviparous cockroach *Nauphoeta cinerea* by Zhu *et al.* (1983). Yin *et al.* (1990) and Lee (1992) have indeed observed significant amounts of ecdysteroids in the haemolymph of another dipteran *P. regina* during the adult stage. Secondly, as mentioned above alternative sources such as the head, thorax and body walls also contribute to the ecdysteroids contained in the female (Bownes *et al.*, 1984).

Furthermore, the data indicate that the declining levels of ecdysteroids, coinciding with the degeneration of the nurse cells and the start of chorion deposition during the first reproductive cycle and before oviposition in *L. cuprina* (Fig. 40), may be due to the process of conjugation and inactivation of ecdysteroids in the oöcytes, as a storage for use in embryogenesis of later generations. As mentioned earlier, radioactive cholesterol injected into adult females of *Schistocerca gregaria* was found incorporated into ecdysteroid conjugates in newly-laid eggs (Isaac and Rees, 1985). Similarly, a sharp decrease of ecdysteroid levels coinciding with chorionation was also observed in the blowfly *C. vomitoria* (Campan *et al.*, 1985). This suggests that this process may occur in all cyclorraphous dipterans.

In addition, the data showed that non-protein fed females of *L. cuprina* did not exhibit a significant increase in ecdysteroid levels. It is only after being fed protein that females displayed an ecdysteroid increase both in the reproductive system and whole bodies (Figs. 43 to 45). This is consistent with the four-hourly timed ecdysteroid levels in developing ovaries of liver-fed flies of the same species (Lefevere *et al.*, 1990). The data suggests that a protein meal induces the ovaries to produce and release ecdysteroids which are important for oogenesis to

proceed. Since ovarian ecdysteroid fluctuations are simultaneous and parallel to those occurring in the haemolymph as observed in the blowfly *C. vomitoria* (Campan *et al.*, 1985), one may conclude that ecdysteroid profiles expressed in whole-body and haemolymph are comparable in this species. The effect of protein feeding on the level of ecdysteroids was also observed in the haemolymph of the black blowfly *P. regina* (Yin *et al.*, 1990). In the latter species an ecdysteroid increase occurred before the increase and peak of the JH, vitellogenin and vitelline. In contrast, in *L. cuprina,* the increase and peak of ovarian ecdysteroids occurred exactly at the same time as that of the JHB_3 *i.e.* 64 h post-eclosion and reached a peak at 68 h (Lefevere *et al.*, 1993). Furthermore, Clissold *et al.* (1993) observed an ecdysteroid peak in thoroughly-staged ovaries at approximately 72 h post-eclosion of *L. cuprina* fed on protein at 24 h and 48 h post eclosion. This 4 h difference in timing of the ecdysteroid peak may be due to the variation in ovarian development at a given age. Apart from these discrepancies of timing between studies, my data in *L. cuprina* support the findings that a protein meal triggers ecdysteroid synthesis as well as secretory activities in both ovaries and corpora allata.

Finally, a mode of action of protein feeding causing ovarian ecdysteroid production has been proposed based on ultrastructural studies in *P. regina,* in which a protein meal causes the release of secretory granules into the haemolymph from the midgut endocrine cells (Stoffolano *et al.*, 1989). This endocrine message from the midgut may stimulate the brain to synthesise, and via the CC release ESG that, in turn, causes the ovaries to produce and release ecdysteroids (Adams *et al.*, 1997). Furthermore, it has been demonstrated in the house fly *Musca domestica* that protein feeding does not increase ecdysteroid

levels if the CA-CC complex or ovaries have been removed (Adams and Gerst, 1992), suggesting that both JH and ESG are required for ovarian ecdysteroid production. However, ESG alone stimulates ovarian development to late vitellogenesis, when injected into flies without the CA-CC complex, and stimulated maximal ovarian ecdysteroid production *in vitro* (Adams *et al.*, 1997). In *L. cuprina,* however, further work is needed to explain the mode of action of protein feeding in causing ovarian ecdysteroid production.

4.11. Adult male development

The trend of whole-body weight in adult males of *L. cuprina* did not differ from that of females, *i.e.* a weight decrease shortly after emergence followed by a significant increase after the protein meal (Fig. 39). However, the increase in weight of males following liver feeding appeared to be reversible, as indicated by the return back, 21 h later, to the weight before liver meal. This may be related to differences in the structure and function of the fat body between males and females. The male adult fat body has relatively large lipid and glycogen stores and little endoplasmic reticulum (*i.e.* in *Schistocerca gregaria,* Odhiambo, 1967 and Stoppie *et al.*, 1981) as compared to females. This suggests that the fat body in adult males is not involved in major protein synthesis, rather it provides reserves of energy needed for very active flying in mate seeking (Bhaktan and Gilbert, 1972). The above may also explain the sexual difference in whole-body weight found here in *L. cuprina.*

4.12. Ecdysteroids in adult males

The presence of detectable amount of ecdysteroids (approximately 10 pg/mg fresh weight) in adult males of *L. cuprina* at emergence, followed by a continuous decrease to a barely detectable level, is in agreement with measurements in whole bodies of *N. bullata* (Wentworth and Roberts, 1984), and *D. melanogaster* (Bownes *et al.*, 1984). Moreover, whole-body data in *L. cuprina* follow the same pattern as in haemolymph of *M. domestica* (Adams *et al.*, 1989) and *P. regina* (Lee, 1992). Since the decrease in ecdysteroid levels following adult emergence is similar to that in females, the mechanisms responsible for this are likely to be the same: *i.e.* excretion of ecdysteroid metabolites through the release of the meconium.

In contrast to the female, a protein meal did not trigger any detectable surge in the level of total ecdysteroids in males of *L. cuprina*. A similar result was found in the haemolymph of other male dipterans, such as *M. domestica* (Adams *et al.*, 1989) and *P. regina* (Lee, 1992). Nevertheless, it is noteworthy that the fat body of both males and females potentially possess similar vitellogenin synthesis capacities when induced by proper and appropriate hormonal cues. Males of *N. bullata* (De Loof *et al.*, 1981) and of *M. domestica* (Adams *et al.*, 1989) are indeed capable of producing vitellogenins after treatment with a juvenile hormone analogue (methoprene) and 20-hydroxyecdysone.

Finally, it is important to note that the very low level of RIA-detectable ecdysteroids in whole-body male dipterans during the reproductive stage does not preclude a critical role of ecdysteroids in reproduction. Indeed, it has been demonstrated in other insects such as the lepidopterans *Heliothis virescens* (Loeb *et al.*, 1982), *Lymantria dispar* (Loeb *et al.*, 1988) and *Ostrinia nubilalis* (Gelman

et al., 1989) that testes sheaths produce ecdysteroids *in vitro* under the induction of a brain peptide (testis ecdysiotropin, TE) which is structurally different from PTTH (Delbecque *et al.*, 1990). It is possible that the testes in Diptera have a similar capacity of ecdysteroid synthesis, which cannot be detected in whole-body extracts (Figs. 46 and 47) or in the haemolymph (Adams *et al.,* 1989; Lee, 1992). Further investigations such as *in vitro* assay of testes are thus required in *L. cuprina.*

Appendix 1

ALCOHOLIC BOUIN'S FIXATIVE

Picric acid (Crystal)	1 g
Formalin (40%)	60 ml
Acetic acid (glacial)	15 ml
Ethanol (80%)	150 ml

ANTISERUM SOLUTION

Before diluting the A-2 ecdysone antiserum an antiserum binding capacity test was performed to determine the concentration of antiserum solution required (see Chapter 2. Materials and Methods). To make up 50 ml of 0.4 % antiserum solution, 200 µl of antiserum was mixed with 200 µl glycerol and placed into a 100 ml reagent bottle. at room temperature 4.8 ml of calf serum (bovine serum albumine) and 44.8 ml of borate buffer was added to the bottle and mixed. The solution was stored ar 4° C until required.

BORATE BUFFER

0.1 M Boric acid	6.184 g
0.1 M Borax	9.536 g
0.075 M NaCl	4.384 g

All compounds were mixed together and made up to 1 litre with distilled H_20, pH was adjusted to 8.4.

CONTROL SERUM

A ten percent solution (v/v) of bovine serum albumine was made in borate buffer.

^{3}H-ECDYSONE SOLUTION

One half millilitres of 0.1 mCi/ml ^{3}H-ecdysone was placed a 1.5 l reagent bottle and dried down in a vacuum dryer. One litre of borate buffer was added into the bottle. To estimate the counts present in the ^{3}H-ecdysone/borate buffer mixture, 25 µl of the solution was placed in a 6 x 50 mm culture tubes. Four hundred microlitres of aquasol-2 was added to the tube and mixed. The sample sample was left in the counter over night before being counted. The radioactivity was adjusted to 80 cpm/µl by evaporating the solution in a vacuum dryer if the count was too low or by adding borate buffer if the count was too high.

NON-RADIOACTIVE ECDYSONE SOLUTIONS

These solutions were used to calculate the standard graphs. To make up a 1 µg/ml of ecdysone solution, 0.1 mg of ecdysone was disolved in 1 ml of 100% ethanol. An optical density reading of the solution was obtained as follows:

1 m/l = 12,800 ImM/ml
ξ_{λ}=242

1 µg/ml = 12.8 O.D.

SATURATED AMMONIUM SULPHATE

Five hundred millilitres of borate buffer was placed into a pyrex beaker on magnetic stirrer at 4° C. Ammonium sulphate $(NH_4)_2SO_4$ (crystal) was added until the solution was saturated.

SECTION OF PUPAE

1. Anaesthetize animals in ether.
2. Puncture the cuticle with a fine needle to allow penetration of fixative.
3. Place animal into fixative and leave for 12 - 24 hours. Use Bouins' fixative or modified Bouins'.
4. Wash material in 70% ETOH until no yellow colour appears.
5. Take the tissues up through a series of gradual Ethanols to 100% dry. Each step for 6 hours. By 10% increases. At least 2 changes in 100%.
6. Place tissue into (10% LR-White 90% dry Ethanol) LR-White/Ethanol. Increase the concentration of LR-White in 10% steps upto 100%, 6 hours per step.
7. Polymerize the resin under UV light and nitrogen.

Appendix 2

Multiple comparisons of the mean values of ecdysteroid titres during embryonic stages of *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

LEVELS ENCOUNTERED DURING PROCESSING ARE:
AGE 0 2 4 6 8 10 12 h

DEP VAR: TITRES N: 35 MULTIPLE R: 0.966 SQUARED MULTIPLE R: 0.934

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	62688.423	6	10448.070	65.823882	0.000
ERROR	4444.374	28	158.727		

COL/ ROW	AGE
1	0
2	2
3	4
4	6
5	8
6	10
7	12

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRES

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	80.611000	0.000000			
3	125.531000	44.920000	0.000000		
4	74.854400	-5.756600	-50.676600	0.000000	
5	47.930600	-32.680400	-77.600400	-26.923800	0.000000
6	17.415600	-63.195400	-108.115400	-57.438800	-30.515000
7	8.582400	-72.028600	-116.948600	-66.272000	-39.348200

	6	7
6	0.000000	
7	-8.833200	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.000145	1.000000			
3	0.000145	0.000220	1.000000		
4	0.000145	0.990032	0.000153	1.000000	
5	0.000169	0.005359	0.000145	0.031225	1.000000
6	0.334522	0.000145	0.000145	0.000145	0.010546
7	0.929716	0.000145	0.000145	0.000145	0.000708

	6	7
6	1.000000	
7	0.920222	1.000000

Appendix 3

Multiple comparison and the Tukey test of hypothesis of mouth hook length during the first instar of *Lucilia cuprina*.

LEVELS ENCOUNTERED DURING PROCESSING ARE:
AGE 0 2 4 6 8 10 12 14 16 18 h

DEP VAR: LENGTH N: 100 MULTIPLE R: 0.820 SQUARED MULTIPLE R: 0.672

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	169.452084	9	18.828009	20.518185	0.000000
ERROR	82.586295	90	0.917625		

COL/ROW	AGE (h)
1	0
2	2
3	4
4	6
5	8
6	10
7	12
8	14
9	16
10	18

USING LEAST SQUARES MEANS.
POST HOC TEST OF LENGTH

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	0.743668	0.000000			
3	1.261003	0.517334	0.000000		
4	1.839085	1.095416	0.578082	0.000000	
5	3.916261	3.172592	2.655258	2.077176	0.000000
6	4.133776	3.390108	2.872774	2.294692	0.217516
7	2.429904	1.686236	1.168901	0.590819	-1.486357
8	1.725428	0.981760	0.464425	-0.113657	-2.190833
9	1.211033	0.467365	-0.049970	-0.628052	-2.705228
10	0.524193	-0.219475	-0.736810	-1.314892	-3.392068

	6	7	8	9	10
6	0.000000				
7	-1.703872	0.000000			
8	-2.408348	-0.704476	0.000000		
9	-2.922743	-1.218871	-0.514395	0.000000	
10	-3.609583	-1.905711	-1.201235	-0.686840	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.772097	1.000000			
3	0.108998	0.969376	1.000000		
4	0.001853	0.253756	0.938885	1.000000	
5	0.000159	0.000159	0.000160	0.000352	1.000000
6	0.000159	0.000159	0.000159	0.000181	0.999965
7	0.000164	0.006096	0.178419	0.930523	0.026388
8	0.004504	0.404673	0.985147	1.000000	0.000222
9	0.143374	0.984486	1.000000	0.901796	0.000159
10	0.966661	0.999962	0.781347	0.079665	0.000159

	6	7	8	9	10
6	1.000000				
7	0.005322	1.000000			
8	0.000166	0.822538	1.000000		
9	0.000159	0.137483	0.970494	1.000000	
10	0.000159	0.001099	0.151014	0.843177	1.000000

Appendix 4

Multiple comparison and the Tukey test of hypothesis of mouth hook length during the second instar of *Lucilia cuprina*.

LEVELS ENCOUNTERED DURING PROCESSING ARE:
AGE 20 22 24 26 28 30 32 34 h

DEP VAR: LENGTH2 N: 80 MULTIPLE R: 0.829 SQUARED MULTIPLE R: 0.687

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	4699.789049	7	671.398436	22.566486	0.000000
ERROR	2142.145094	72	29.752015		

COL/ROW	AGE (h)
1	20
2	22
3	24
4	26
5	28
6	30
7	32
8	34

USING LEAST SQUARES MEANS.
POST HOC TEST OF LENGTH2

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	2.378400	0.000000			
3	13.171200	10.792800	0.000000		
4	12.801600	10.423200	-0.369600	0.000000	
5	-3.528000	-5.906400	-16.699200	-16.329600	0.000000
6	-5.901600	-8.280000	-19.072800	-18.703200	-2.373600
7	-6.177600	-8.556000	-19.348800	-18.979200	-2.649600
8	-6.780000	-9.158400	-19.951200	-19.581600	-3.252000

	6	7	8
6	0.000000		
7	-0.276000	0.000000	
8	-0.878400	-0.602400	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.976626	1.000000			
3	0.000140	0.000946	1.000000		
4	0.000156	0.001533	1.000000	1.000000	
5	0.832438	0.247237	0.000123	0.000123	1.000000
6	0.248156	0.023675	0.000123	0.000123	0.976890
7	0.198952	0.017063	0.000123	0.000123	0.957764
8	0.116625	0.008056	0.000123	0.000123	0.883113

	6	7	8
6	1.000000		
7	1.000000	1.000000	
8	0.999962	0.999997	1.000000

Appendix 5

Multiple comparison and the Tukey test of hypothesis of mouth hook length during the third instar of *Lucilia curpina.*

LEVELS ENCOUNTERED DURING PROCESSING ARE:
AGE 36 38 40 42 44 52 h

DEP VAR: LENGTH N: 60 MULTIPLE R: 0.585 SQUARED MULTIPLE R: 0.343

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	5262.790205	5	1052.558041	5.629469	0.000302
ERROR	.100965E+05	54	186.972871		

COL/ROW	AGE (h)
1	36
2	38
3	40
4	42
5	44
6	52

USING LEAST SQUARES MEANS.
POST HOC TEST OF LENGTH3

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	4.816800	0.000000			
3	12.436800	7.620000	0.000000		
4	16.584000	11.767200	4.147200	0.000000	
5	23.685600	18.868800	11.248800	7.101600	0.000000
6	25.960800	21.144000	13.524000	9.376800	2.275200

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.968517	1.000000			
3	0.337444	0.812178	1.000000		
4	0.089466	0.399100	0.983689	1.000000	
5	0.003877	0.035891	0.449979	0.852980	1.000000
6	0.001271	0.013102	0.249590	0.644747	0.999071

Appendix 6

Analysis of variance and regression for body weight measurements of the first instar larvae (0 - 18 h) of *Luciiia cuprina.*

Regression statistics

Multiple R	0.72
R Square	0.51
Adjusted R Square	0.50
Standard Error	0.11
Observations	40.00

Analysis of Variance

	df	Sum of Squares	Mean Square	F	Significance F
Regression	1	0.48	0.48	39.84	0.000000
Residual	38	0.46	0.01		
Total	39	0.94			

	Coefficients	Standard Error	t Statistic	P-value	Lower 95%	Upper 95%
Intercept	-0.04	0.04	-1.02	.312190	-0.11	0.04
Age	0.02	0	6.31	.000000	0.01	0.03

Appendix 7

Analysis of variance and regression for body weight measurements of the second instar larvae (18 - 34 h) of *Lucilia cuprina*.

Regression statistics

Multiple R	0.72
R Square	0.51
Adjusted R Square	0.50
Standard Error	0.11
Observations	40

Analysis of Variance

	df	Sum of Squares	Mean Square	F	Significance F
Regression	1	0.48	0.48	39.84	0.000000
Residual	38	0.46	0.01		
Total	39	0.94			

	Coefficients	Standard Error	t Statistic	P-value	Lower 95%	Upper 95%
Intercept	-0.04	0.04	-1.02	0.312190	-0.11	0.04
Age	0.02	0	6.31	0.000000	0.01	0.03

Appendix 8

Analysis of variance and regression for body weight measurements of the third instar larvae of *Lucilia cuprina*.

Feeding stage

Regression statistics	
Multiple R	0.72
R Square	0.52
Adjusted R Square	0.52
Standard Error	10.3
Observations	85

Analysis of Variance

	df	Sum of Squares	Mean Square	F	Significance F
Regression	1	9704.54	9704.54	91.52	0.000000
Residual	83	8801.54	106.04		
Total	84	18506.08			

	Coefficients	Standard Error	t Statistic	P-value	Lower 95%	Upper 95%
Intercept	5.14	3.82	1.35	0.18	-2.45	12.73
Age	0.57	0.06	9.57	0.000000	0.46	0.69

Post-feeding stage

Regression statistics	
Multiple R	0.35
R Square	0.12
Adjusted R Square	0.10
Standard Error	5.34
Observations	45

Analysis of Variance

	df	Sum of Squares	Mean Square	F	Significance F
Regression	1	170.47	170.47	5.97	0.02
Residual	43	1227.22	28.54		
Total	44	1397.69			

	Coefficients	Standard Error	t Statistic	P-value	Lower 95%	Upper 95%
Intercept	66.06	8.37	7.9	0.0000000	49.19	82.94
Age	-0.19	0.08	-2.44	0.02	-0.34	-0.03

Appendix 9

Multiple comparisons of the mean values of ecdysteroid titres during the first instar larvae of *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

LEVELS ENCOUNTERED DURING PROCESSING ARE:
AGE 0 4 8 10 12 14 16 18 h

DEP VAR: TITRES N: 40 MULTIPLE R: 0.940 SQUARED MULTIPLE R: 0.883

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	33648.948	7	4806.993	34.401	0.000
ERROR	4471.513	32	139.735		

COL/ROW	AGE
1	0
2	4
3	8
4	10
5	12
6	14
7	16
8	18

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRES

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	-22.798800	0.000000			
3	49.862800	72.661600	0.000000		
4	25.210200	48.009000	-24.652600	0.000000	
5	-20.108000	2.690800	-69.970800	-45.318200	0.000000
6	-32.803000	-10.004200	-82.665800	-58.013200	-12.695000
7	-35.453200	-12.654400	-85.316000	-60.663400	-15.345200
8	-29.927200	-7.128400	-79.790000	-55.137400	-9.819200

	6	7	8
6	0.000000		
7	-2.650200	0.000000	
8	2.875800	5.526000	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.076947	1.000000			
3	0.000140	0.000138	1.000000		
4	0.036659	0.000142	0.043734	1.000000	
5	0.162737	0.999956	0.000138	0.000154	1.000000
6	0.002720	0.877261	0.000138	0.000138	0.688410
7	0.001096	0.691737	0.000138	0.000138	0.465074
8	0.007493	0.977808	0.000138	0.000138	0.987097

	6	7	8
6	1.000000		
7	0.999960	1.000000	
8	0.999931	0.995014	1.000000

Appendix 10

Multiple comparisons of the mean values of ecdysteroid titres during the second instar larvae of *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

LEVELS ENCOUNTERED DURING PROCESSING ARE:

AGE 18 20 22 24 26 28 32 34 h

DEP VAR: TITRES N: 40 MULTIPLE R: 0.976 SQUARED MULTIPLE R: 0.952

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	11626.110	7	1660.873	90.366	0.000
ERROR	588.139	32	18.379		

COL/ROW	AGE
1	18
2	20
3	22
4	24
5	26
6	28
7	32
8	34

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRES

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	2.174800	0.000000			
3	0.570400	-1.604400	0.000000		
4	12.686200	10.511400	12.115800	0.000000	
5	42.280600	40.105800	41.710200	29.594400	0.000000
6	-8.012400	-10.187200	-8.582800	-20.698600	-50.293000
7	-12.213800	-14.388600	-12.784200	-24.900000	-54.494400
8	-14.898800	-17.073600	-15.469200	-27.585000	-57.179400

	6	7	8
6	0.000000		
7	-4.201400	0.000000	
8	-6.886400	-2.685000	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.991830	1.000000			
3	0.999999	0.998787	1.000000		
4	0.001283	0.010407	0.002203	1.000000	
5	0.000138	0.000138	0.000138	0.000138	1.000000
6	0.094488	0.014143	0.059348	0.000138	0.000138
7	0.002006	0.000319	0.001172	0.000138	0.000138
8	0.000237	0.000145	0.000189	0.000138	0.000138

	6	7	8
6	1.000000		
7	0.775267	1.000000	
8	0.215693	0.972652	1.000000

Appendix 11

Multiple comparisons of the mean values of ecdysteroid titres during the feeding stage of the third instar larvae of *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

LEVELS ENCOUNTERED DURING PROCESSING ARE:

AGE	34	36	38	40	44	48	52	56	60	64	68
	72	76	80	84	88	92	96	100	h		

DEP VAR: TITRES N: 95 MULTIPLE R: 0.827 SQUARED MULTIPLE R: 0.683

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	5358.683898	18	297.704661	9.113063	0.000
ERROR	2482.760758	76	32.667905		

COL/ROW	AGE
1	34
2	36
3	38
4	40
5	44
6	48
7	52
8	56
9	60
10	64
11	68
12	72
13	76
14	80
15	84
16	88
17	92
18	96
19	100

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRES

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	-1.700800	0.000000			
3	6.046600	7.747400	0.000000		
4	0.657000	2.357800	-5.389600	0.000000	
5	17.363400	19.064200	11.316800	16.706400	0.000000
6	4.032600	5.733400	-2.014000	3.375600	-13.330800
7	4.280000	5.980800	-1.766600	3.623000	-13.083400
8	16.291400	17.992200	10.244800	15.634400	-1.072000
9	14.800000	16.500800	8.753400	14.143000	-2.563400
10	3.624400	5.325200	-2.422200	2.967400	-13.739000
11	5.917800	7.618600	-0.128800	5.260800	-11.445600
12	7.536600	9.237400	1.490000	6.879600	-9.826800
13	12.251800	13.952600	6.205200	11.594800	-5.111600
14	6.309600	8.010400	0.263000	5.652600	-11.053800
15	14.379200	16.080000	8.332600	13.722200	-2.984200
16	11.501200	13.202000	5.454600	10.844200	-5.862200
17	17.407200	19.108000	11.360600	16.750200	0.043800
18	12.020800	13.721600	5.974200	11.363800	-5.342600
19	29.680600	31.381400	23.634000	29.023600	12.317200

	6	7	8	9	10
6	0.000000				
7	0.247400	0.000000			
8	12.258800	12.011400	0.000000		
9	10.767400	10.520000	-1.491400	0.000000	
10	-0.408200	-0.655600	-12.667000	-11.175600	0.000000
11	1.885200	1.637800	-10.373600	-8.882200	2.293400
12	3.504000	3.256600	-8.754800	-7.263400	3.912200
13	8.219200	7.971800	-4.039600	-2.548200	8.627400
14	2.277000	2.029600	-9.981800	-8.490400	2.685200
15	10.346600	10.099200	-1.912200	-0.420800	10.754800
16	7.468600	7.221200	-4.790200	-3.298800	7.876800
17	13.374600	13.127200	1.115800	2.607200	13.782800
18	7.988200	7.740800	-4.270600	-2.779200	8.396400
19	25.648000	25.400600	13.389200	14.880600	26.056200

	11	12	13	14	15
11	0.000000				
12	1.618800	0.000000			
13	6.334000	4.715200	0.000000		
14	0.391800	-1.227000	-5.942200	0.000000	
15	8.461400	6.842600	2.127400	8.069600	0.000000
16	5.583400	3.964600	-0.750600	5.191600	-2.878000
17	11.489400	9.870600	5.155400	11.097600	3.028000
18	6.103000	4.484200	-0.231000	5.711200	-2.358400
19	23.762800	22.144000	17.428800	23.371000	15.301400

	16	17	18	19
16	0.000000			
17	5.906000	0.000000		
18	0.519600	-5.386400	0.000000	
19	18.179400	12.273400	17.659800	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	1.000000	1.000000			
3	0.972677	0.804205	1.000000		
4	1.000000	1.000000	0.991603	1.000000	
5	0.001232	0.000343	0.183552	0.002238	1.000000
6	0.999771	0.983855	1.000000	0.999982	0.044047
7	0.999493	0.975413	1.000000	0.999949	0.053435
8	0.003300	0.000724	0.334985	0.006061	1.000000
9	0.012851	0.002709	0.620165	0.022674	1.000000
10	0.999948	0.992649	1.000000	0.999997	0.031719
11	0.977837	0.823991	1.000000	0.993576	0.169396
12	0.836080	0.523253	1.000000	0.915595	0.409047
13	0.098828	0.026598	0.965156	0.154046	0.955362
14	0.959426	0.760358	1.000000	0.986053	0.215138
15	0.018546	0.004017	0.701931	0.032158	0.999997
16	0.163546	0.048735	0.990440	0.242922	0.979808
17	0.001185	0.000335	0.178643	0.002149	1.000000
18	0.116019	0.032173	0.975674	0.178288	0.992376
19	0.000170	0.000170	0.000170	0.000170	0.094363

	6	7	8	9	10
6	1.000000				
7	1.000000	1.000000			
8	0.098342	0.116767	1.000000		
9	0.253686	0.290517	1.000000	1.000000	
10	1.000000	1.000000	0.073248	0.200058	1.000000
11	1.000000	1.000000	0.313712	0.594449	1.000000
12	0.999968	0.999989	0.619887	0.872979	0.999849
13	0.723012	0.767025	0.999766	1.000000	0.645099
14	1.000000	1.000000	0.380746	0.671822	0.999999
15	0.318107	0.359948	1.000000	1.000000	0.255481
16	0.845757	0.878199	0.997864	0.999987	0.783084
17	0.042547	0.051655	1.000000	1.000000	0.030601
18	0.764203	0.805150	0.999508	0.999999	0.689857
19	0.000170	0.000170	0.042057	0.011970	0.000170

	11	12	13	14	15
11	1.000000				
12	1.000000	1.000000			
13	0.957990	0.998246	1.000000		
14	1.000000	1.000000	0.976917	1.000000	
15	0.677414	0.919132	1.000000	0.749984	1.000000
16	0.987733	0.999818	1.000000	0.994466	0.999998
17	0.164775	0.400957	0.994888	0.209623	0.999996
18	0.970159	0.999075	1.000000	0.984486	1.000000
19	0.000170	0.000174	0.001163	0.000170	0.008202

	16	17	18	19
16	1.000000			
17	0.978268	1.000000		
18	1.000000	0.991657	1.000000	
19	0.000627	0.097334	0.000952	1.000000

Appendix 12

Multiple comparisons of the mean values of ecdysteroid titres during the post-feeding stage of the third instar larvae of *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

LEVELS ENCOUNTERED DURING PROCESSING ARE:

AGE 96 100 104 108 112 116 120 124 128 h

DEP VAR: TITRE N: 45 MULTIPLE R: 0.939 SQUARED MULTIPLE R: 0.881

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	363346.070	8	45418.259	33.370	0.000
ERROR	48997.385	36	1361.038		

COL/ROW	AGE
1	96
2	100
3	104
4	108
5	112
6	116
7	120
8	124
9	128

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRE

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	17.661600	0.000000			
3	5.102400	-12.559200	0.000000		
4	6.244000	-11.417600	1.141600	0.000000	
5	21.311200	3.649600	16.208800	15.067200	0.000000
6	15.019000	-2.642600	9.916600	8.775000	-6.292200
7	56.894800	39.233200	51.792400	50.650800	35.583600
8	201.955800	184.294200	196.853400	195.711800	180.644600
9	223.543800	205.882200	218.441400	217.299800	202.232600

	6	7	8	9
6	0.000000			
7	41.875800	0.000000		
8	186.936800	145.061000	0.000000	
9	208.524800	166.649000	21.588000	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.998057	1.000000			
3	1.000000	0.999842	1.000000		
4	0.999999	0.999923	1.000000	1.000000	
5	0.992871	1.000000	0.998949	0.999393	1.000000
6	0.999407	1.000000	0.999974	0.999990	0.999999
7	0.336390	0.787246	0.460925	0.490892	0.862019
8	0.000143	0.000143	0.000143	0.000143	0.000143
9	0.000143	0.000143	0.000143	0.000143	0.000143

	6	7	8	9
6	1.000000			
7	0.724153	1.000000		
8	0.000143	0.000153	1.000000	
9	0.000143	0.000143	0.980462	1.000000

Appendix 13

Comparison between female and male weights of *Lucilia cuprina* during white-prepupal stage using t-Test.

	Female	Male
Mean	46.17	36.34
Standard Error	0.23	0.22
Median	46.16	36.61
Mode	48.09	37.68
Standard Deviation	3.05	2.55
Variance	9.32	6.53
Kurtosis	-0.26	0.7
Skewness	0.07	-0.84
Range	17.12	13.13
Minimum	39.02	27.65
Maximum	56.14	40.78
Sum	8449.97	4833.76
Count	183	133

t-Test: Two-Sample Assuming Unequal Variances

	Female	Male
Mean	46.17	36.34
Variance	9.32	6.53
Observations	183	133
Pooled Variance	3.5	
df	307.74	
t	31.09	
P(T<=t) one-tail	0	
t Critical one-tail	1.65	
P(T<=t) two-tail	0	
t Critical two-tail	1.97	

Appendix 14

Analysis of variance and regression for body weight measurements of females and males *Lucilia cuprina* throughout prepupal stages (0 - 8 h).

Female

Regression analysis	
Multiple R	0.7
R Square	0.49
Adjusted R Square	0.448274
Standard Error	2.47
Observations	15

Analysis of Variance

	df	Sum of Squares	Mean Square	F	Significance F
Regression	1	75.63	75.63	12.37	0
Residual	13	79.44	6.11		
Total	14	155.07			

	Coefficients	Standard Error	t Statistic	P-value	Lower 95%	Upper 95%
Intercept	49.91	1.01	49.45	0.000000	47.73	52.09
Age	-0.69	0.2	-3.52	0	-1.11	-0.27

Male

Regression Statistics	
Multiple R	0.3
R Square	0.09
Adjusted R Square	0.02
Standard Error	3.73
Observations	15

Analysis of Variance

	df	Sum of Squares	Mean Square	F	Significance F
Regression	1	17.34	17.34	1.25	0.28
Residual	13	180.43	13.88		
Total	14	197.77			

	Coefficients	Standard Error	t Statistic	P-value	Lower 95%	Upper 95%
Intercept	34.41	1.52	22.62	0.000000	31.12	37.69
Age	-0.33	0.29	-1.12	0.28	-0.97	0.31

Appendix 15

Analysis of variance and regression for body weight measurements of females and males *Lucilia cuprina* throughout pupal stages (8 - 36 h).

Female

Regression Statistics	
Multiple R	0.37
R Square	0.13
Adjusted R Square	0.111382
Standard Error	1.54
Observations	40

Analysis of Variance

	df	Sum of Squares	Mean Square	F	Significance F
Regression	1	13.93	13.93	5.89	0.02
Residual	38	89.88	2.37		
Total	39	103.8			

	Coefficients	Standard Error	t Statistic	P-value	Lower 95%	Upper 95%
Intercept	44.37	0.63	70.17	0.000000	43.09	45.65
Age	-0.06	0.03	-2.43	0.02	-0.12	-0.01

Male

Regression Statistics	
Multiple R	0.13
R Square	0.02
Adjusted R Square	-0.01
Standard Error	2.39
Observations	40

Analysis of Variance

	df	Sum of Squares	Mean Square	F	Significance F
Regression	1	4.01	4.01	0.7	0.41
Residual	38	216.42	5.7		
Total	39	220.43			

	Coefficients	Standard Error	t Statistic	P-value	Lower 95%	Upper 95%
Intercept	32.59	0.98	33.21	0.000000	30.6	34.58
Age	0.03	0.04	0.84	0.41	-0.05	0.12

Appendix 16

Analysis of variance and regression for body weight measurements of females and males *Lucilia cuprina* throughout pharate-adult stages (36 - 160 h).

Female

Regression Statistics	
Multipie R	0.45
R Square	0.2
Adjusted R Square	0.2
Standard Error	1.93
Observations	160

Analysis of Variance

	df	Sum of Squares	Mean Square	F	Significance F
Regression	1	146.8	146.8	39.56	0.000000
Residual	158	586.34	3.71		
Total	159	733.14			

	Coefficients	Standard Error	t Statistic	P-value	Lower 95%	Upper 95%
Intercept	43.98	0.43	101.83	0.000000	43.12	44.83
Age	-0.03	0	-6.29	0.000000	-0.03	-0.02

Male

Regression Statistics	
Multiple R	0.33
R Square	0.11
Adjusted R Square	0.106021
Standard Error	2.16
Observations	160

Analysis of Variance

	df	Sum of Squares	Mean Square	F	Significance F
Regression	1	92.43	92.43	19.86	0
Residual	158	735.48	4.65		
Total	159	827.91			

	Coefficients	Standard Error	t Statistic	P-value	Lower 95%	Upper 95%
Intercept	33.49	0.48	69.25	0.000000	32.54	34.45
Age	-0.02	0	-4.46	0	-0.03	-0.01

Appendix 17

Analysis of variance and regression for body weight measurements of females and males *Lucilia cuprina* throughout prepupal, pupal and pharate-adult stages (0 - 160 h).

Female

Regression Statistics	
Multiple R	0.56
R Square	0.32
Adjusted R Square	0.313945
Standard Error	2.18
Observations	205

Analysis of Variance

	df	Sum of Squares	Mean Square	F	Significance F
Regression	1	448.49	448.49	94.35	0.000000
Residual	203	964.93	4.75		
Total	204	1413.41			

	Coefficients	Standard Error	t Statistic	P-value	Lower 95%	Upper 95%
Intercept	44.52	0.3	148.86	0.000000	43.93	45.11
Age	-0.03	0	-9.71	0.000000	-0.04	-0.02

Male

Regression Statistics	
Multiple R	0.38
R Square	0.14
Adjusted R Square	0.140579
Standard Error	2.36
Observations	205

Analysis of Variance

	df	Sum of Squares	Mean Square	F	Significance F
Regression	1	191.53	191.53	34.37	0.000000
Residual	203	1131.28	5.57		
Total	204	1322.81			

	Coefficients	Standard Error	t Statistic	P-value	Lower 95%	Upper 95%
Intercept	33.49	0.32	103.42	0.000000	32.85	34.13
Age	-0.02	0	-5.86	0.000000	-0.03	-0.01

Appendix 18

Comparison between female and male weights of *Lucilia cuprina* throughout prepupal, pupal and pharate adult stages using t-Test.

	Female	Male
Mean	42.02	32.26
Std Dev	2.63	2.76
Std Err	0.18	0.19
95% Conf	0.36	0.38
99% Conf	0.48	0.5
Size	205	205
Sum	8613.43	6612.3
Max	53.63	39.73
Min	32.48	23.46

t-Test: Paired Two-Sample for Means

	Female	Male
Mean	42.02	32.26
Variance	6.93	7.62
Observations	205	205
Pearson Correlation	0.45	
Pooled Variance	3.27	
Hypothesized Mean Difference	0	
df	204	
t	49.42	
P(T<=t) one-tail	0.000000	
t Critical one-tail	1.65	
P(T<=t) two-tail	0.000000	
t Critical two-tail	1.97	

Appendix 19

Multiple comparisons of the mean values of ecdysteroid titres in female prepupae of *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

LEVELS ENCOUNTERED DURING PROCESSING ARE:
AGE 0 4 8 h

DEP VAR: TITRE N: 15 MULTIPLE R: 0.859 SQUARED MULTIPLE R: 0.738

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	47898.099	2	23949.049	16.884	0.000
ERROR	17021.806	12	1418.484		

COL/ROW	AGE
1	0
2	4
3	8

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRE

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3
1	0.000000		
2	71.867600	0.000000	
3	-66.514800	-138.382400	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3
1	1.000000		
2	0.026999	1.000000	
3	0.040250	0.000390	1.000000

Appendix 20

Multiple comparisons of the mean values of ecdysteroid titres in male prepupae of *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

LEVELS ENCOUNTERED DURING PROCESSING ARE:
AGE 0 4 8 h

DEP VAR: TITRE N: 15 MULTIPLE R: 0.775 SQUARED MULTIPLE R: 0.600

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	87385.656	2	43692.828	9.010	0.004
ERROR	58189.617	12	4849.135		

COL/ROW	AGE
1	0
2	4
3	8

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRE

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3
1	0.000000		
2	80.736400	0.000000	
3	-105.669200	-186.405600	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3
1	1.000000		
2	0.020810	1.000000	
3	0.079825	0.003225	1.000000

Appendix 21

Comparison between ecdysteroid titres during prepupal stage in the females and males of *Lucilia cuprina* using t-Test.

	Female	Male
Mean	174.15	194.19
Standard Error	19.67	28.78
Median	179.17	175.84
Standard Deviation	87.97	128.71
Variance	7738.8	16566.68
Kurtosis	-0.46	2.85
Skewness	0.35	1.54
Range	305.09	506.33
Minimum	58.61	64.72
Maximum	363.7	571.05
Sum	3483.01	3883.7
Count	20	20

t-Test: Paired Two-Sample for Means

	Female	Male
Mean	174.15	194.19
Variance	7738.8	16566.68
Observations	20	20
Pearson Correlation	0.75	
Pooled Variance	8511.07	
Hypothesized Mean Difference	0	
df	19	
t	1.05	
P(T<=t) one-tail	0.15	
t Critical one-tail	1.73	
P(T<=t) two-tail	0.31	
t Critical two-tail	2.09	

Appendix 22

Comparison between female and male ecdysteroid peaks during prepupal stage of *Lucilia cuprina* at 4 h using t-Test.

t-Test: Two-Sample for Means

	Female	Male
Mean	281.21	346.14
Variance	3582.34	22611.95
Observations	5	5
Pearson Correlation	-0.18	
Pooled Variance	-1603.74	
Hypothesized Mean Difference	0	
df	4	
t	0.85	
P(T<=t) one-tail	0.22	
t Critical one-tail	2.13	
P(T<=t) two-tail	0.44	
t Critical two-tail	2.78	

Appendix 23

Multiple comparisons of the mean values of ecdysteroid titres in female pupae of *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

LEVELS ENCOUNTERED DURING PROCESSING ARE:
AGE 12 16 20 24 28 32 36 h

DEP VAR: TITRE N: 35 MULTIPLE R: 0.898 SQUARED MULTIPLE R: 0.806

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	30450.122	6	5075.020	19.343	0.000
ERROR	7346.230	28	262.365		

COL/ROW	AGE
1	12
2	16
3	20
4	24
5	28
6	32
7	36

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRE

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	-13.176200	0.000000			
3	4.620400	17.796600	0.000000		
4	3.516600	16.692800	-1.103800	0.000000	
5	23.780000	36.956200	19.159600	20.263400	0.000000
6	59.826600	73.002800	55.206200	56.310000	36.046600
7	69.909400	83.085600	65.289000	66.392800	46.129400

	6	7
6	0.000000	
7	10.082800	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.852479	1.000000			
3	0.999289	0.598338	1.000000		
4	0.999852	0.665408	1.000000	1.000000	
5	0.269501	0.018149	0.515184	0.449827	1.000000
6	0.000186	0.000145	0.000305	0.000258	0.022447
7	0.000147	0.000145	0.000153	0.000151	0.001966

	6	7
6	1.000000	
7	0.953386	1.000000

Appendix 24

Multiple comparisons of the mean values of ecdysteroid titres in male pupae of *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

LEVELS ENCOUNTERED DURING PROCESSING ARE:
AGE 12 16 20 24 28 32 36 h

DEP VAR: TITRE N: 35 MULTIPLE R: 0.939 SQUARED MULTIPLE R: 0.881

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	65675.844	6	10945.974	34.533	0.000
ERROR	8875.253	28	316.973		

COL/ROW	AGE
1	12
2	16
3	20
4	24
5	28
6	32
7	36

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRE

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	-17.596000	0.000000			
3	21.901200	39.497200	0.000000		
4	15.199600	32.795600	-6.701600	0.000000	
5	9.301800	26.897800	-12.599400	-5.897800	0.000000
6	64.520400	82.116400	42.619200	49.320800	55.218600
7	119.813600	137.409600	97.912400	104.614000	110.511800

	6	7
6	0.000000	
7	55.293200	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.705853	1.000000			
3	0.469531	0.023044	1.000000		
4	0.822614	0.087996	0.996496	1.000000	
5	0.980111	0.240521	0.916927	0.998295	1.000000
6	0.000202	0.000145	0.011773	0.002661	0.000763
7	0.000145	0.000145	0.000145	0.000145	0.000145

	6	7
6	1.000000	
7	0.000752	1.000000

Appendix 25

Comparison between ecdysteroid titres during pupal stage in the females and males of *Lucilia cuprina* using t-Test.

	Female	Male
Mean	104.06	121.4
Standard Error	10.29	10.23
Median	81.2	95.93
Standard Deviation	65.07	64.69
Variance	4233.97	4184.37
Kurtosis	3.57	-0.27
Skewness	1.9	0.97
Range	284.3	230.79
Minimum	42.28	51.59
Maximum	326.57	282.38
Sum	4162.51	4856.04
Count	40	40

t-Test: Paired Two-Sample for Means

	Female	Male
Mean	104.06	121.4
Variance	4233.97	4184.37
Observations	40	40
Pearson Correlation	0.81	
Pooled Variance	3403.26	
Hypothesized Mean Difference	0	
df	39	
t	2.73	
P(T<=t) one-tail	0	
t Critical one-tail	1.68	
P(T<=t) two-tail	0.01	
t Critical two-tail	2.02	

Appendix 26

Multiple comparisons of the mean values of ecdysteroid titres in female pharate-adults of *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

LEVELS ENCOUNTERED DURING PROCESSING ARE:

AGE	40	44	48	52	56	60	64	68	72	76	80
		84	88	92	96	100	104	h			

DEP VAR: TITRE N: 80 MULTIPLE R: 0.920 SQUARED MULTIPLE R: 0.846

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	2074524.004	16	138301.600	23.460	0.000
ERROR	377287.293	64	5895.114		

COL/ROW	AGE
1	40
2	44
3	48
4	52
5	56
6	60
7	64
8	68
9	72
10	76
11	80
12	84
13	88
14	92
15	96
16	100
17	104

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRE

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	170.806800	0.000000			
3	248.999400	78.192600	0.000000		
4	335.618000	164.811200	86.618600	0.000000	
5	302.567800	131.761000	53.568400	-33.050200	0.000000
6	457.246800	286.440000	208.247400	121.628800	154.679000
7	411.902200	241.095400	162.902800	76.284200	109.334400
8	468.976200	298.169400	219.976800	133.358200	166.408400
9	358.031400	187.224600	109.032000	22.413400	55.463600
10	298.749800	127.943000	49.750400	-36.868200	-3.818000
11	139.122200	-31.684600	-109.877200	-196.495800	-163.445600
12	83.157200	-87.649600	-165.842200	-252.460800	-219.410600
13	7.930200	-162.876600	-241.069200	-327.687800	-294.637600
14	405.307000	234.500200	156.307600	69.689000	102.739200
15	319.667200	148.860400	70.667800	-15.950800	17.099400
16	-27.847800	-198.654600	-276.847200	-363.465800	-330.415600
17	-29.061400	-199.868200	-278.060800	-364.679400	-331.629200

	6	7	8	9	10
6	0.000000				
7	-45.344600	0.000000			
8	11.729400	57.074000	0.000000		
9	-99.215400	-53.870800	-110.944800	0.000000	
10	-158.497000	-113.152400	-170.226400	-59.281600	0.000000
11	-318.124600	-272.780000	-329.854000	-218.909200	-159.627600
12	-374.089600	-328.745000	-385.819000	-274.874200	-215.592600
13	-449.316600	-403.972000	-461.046000	-350.101200	-290.819600
14	-51.939800	-6.595200	-63.669200	47.275600	106.557200
15	-137.579600	-92.235000	-149.309000	-38.364200	20.917400
16	-485.094600	-439.750000	-496.824000	-385.879200	-326.597600
17	-486.308200	-440.963600	-498.037600	-387.092800	-327.811200

	11	12	13	14	15
11	0.000000				
12	-55.965000	0.000000			
13	-131.192000	-75.227000	0.000000		
14	266.184800	322.149800	397.376800	0.000000	
15	180.545000	236.510000	311.737000	-85.639800	0.000000
16	-166.970000	-111.005000	-35.778000	-433.154800	-347.515000
17	-168.183600	-112.218600	-36.991600	-434.368400	-348.728600

	16	17
16	0.000000	
17	-1.213600	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.047675	1.000000			
3	0.000339	0.961288	1.000000		
4	0.000167	0.067253	0.912312	1.000000	
5	0.000169	0.324631	0.999258	0.999999	1.000000
6	0.000167	0.000173	0.004223	0.462330	0.115781
7	0.000167	0.000492	0.074762	0.968766	0.645925
8	0.000167	0.000169	0.001869	0.305178	0.061470
9	0.000167	0.017357	0.650387	1.000000	0.998873
10	0.000169	0.373825	0.999698	0.999994	1.000000
11	0.240918	0.999999	0.637888	0.009432	0.072556
12	0.935792	0.904367	0.063467	0.000297	0.001943
13	1.000000	0.074870	0.000493	0.000167	0.000170
14	0.000167	0.000722	0.106438	0.986534	0.739709
15	0.000168	0.154692	0.984585	1.000000	1.000000
16	1.000000	0.008157	0.000181	0.000167	0.000167
17	1.000000	0.007500	0.000180	0.000167	0.000167

	6	7	8	9	10
6	1.000000				
7	0.999907	1.000000			
8	1.000000	0.998420	1.000000		
9	0.785512	0.999206	0.621987	1.000000	
10	0.094871	0.588857	0.049318	0.997561	1.000000
11	0.000168	0.000188	0.000167	0.002012	0.089321
12	0.000167	0.000167	0.000167	0.000184	0.002541
13	0.000167	0.000167	0.000167	0.000167	0.000171
14	0.999488	1.000000	0.994641	0.999841	0.686420
15	0.257150	0.863633	0.151379	0.999990	1.000000
16	0.000167	0.000167	0.000167	0.000167	0.000167
17	0.000167	0.000167	0.000167	0.000167	0.000167

	11	12	13	14	15
11	1.000000				
12	0.998748	1.000000			
13	0.331728	0.972412	1.000000		
14	0.000204	0.000167	0.000167	1.000000	
15	0.026484	0.000641	0.000168	0.919448	1.000000
16	0.059544	0.621088	0.999996	0.000167	0.000167
17	0.055559	0.602904	0.999994	0.000167	0.000167

	16	17
16	1.000000	
17	1.000000	1.000000

LEVELS ENCOUNTERED DURING PROCESSING ARE:

AGE	104	108	112	116	120	124	128	132	136	140	144
		148	152	156	160	h					

DEP VAR: TITRE N: 75 MULTIPLE R: 0.811 SQUARED MULTIPLE R: 0.657

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	175389.353	14	12527.811	8.224	0.000
ERROR	91400.557	60	1523.343		

COL/ROW	AGE
1	104
2	108
3	112
4	116
5	120
6	124
7	128
8	132
9	136
10	140
11	144
12	148
13	152
14	156
15	160

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRE

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	-38.189000	0.000000			
3	-89.127000	-50.938000	0.000000		
4	-79.325400	-41.136400	9.801600	0.000000	
5	-83.721600	-45.532600	5.405400	-4.396200	0.000000
6	-98.888800	-60.699800	-9.761800	-19.563400	-15.167200
7	-54.879200	-16.690200	34.247800	24.446200	28.842400
8	-116.373400	-78.184400	-27.246400	-37.048000	-32.651800
9	-117.181400	-78.992400	-28.054400	-37.856000	-33.459800
10	-124.793600	-86.604600	-35.666600	-45.468200	-41.072000
11	-145.820400	-107.631400	-56.693400	-66.495000	-62.098800
12	-156.398200	-118.209200	-67.271200	-77.072800	-72.676600
13	-164.606800	-126.417800	-75.479800	-85.281400	-80.885200
14	-165.272400	-127.083400	-76.145400	-85.947000	-81.550800
15	-164.593800	-126.404800	-75.466800	-85.268400	-80.872200

	6	7	8	9	10
6	0.000000				
7	44.009600	0.000000			
8	-17.484600	-61.494200	0.000000		
9	-18.292600	-62.302200	-0.808000	0.000000	
10	-25.904800	-69.914400	-8.420200	-7.612200	0.000000
11	-46.931600	-90.941200	-29.447000	-28.639000	-21.026800
12	-57.509400	-101.519000	-40.024800	-39.216800	-31.604600
13	-65.718000	-109.727600	-48.233400	-47.425400	-39.813200
14	-66.383600	-110.393200	-48.899000	-48.091000	-40.478800
15	-65.705000	-109.714600	-48.220400	-47.412400	-39.800200

	11	12	13	14	15
11	0.000000				
12	-10.577800	0.000000			
13	-18.786400	-8.208600	0.000000		
14	-19.452000	-8.874200	-0.665600	0.000000	
15	-18.773400	-8.195600	0.013000	0.678600	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.963301	1.000000			
3	0.040915	0.752405	1.000000		
4	0.113460	0.934981	1.000000	1.000000	
5	0.073155	0.869825	1.000000	1.000000	1.000000
6	0.013046	0.483917	1.000000	0.999966	0.999999
7	0.647742	0.999995	0.985486	0.999540	0.997244
8	0.001391	0.126484	0.998484	0.971356	0.990634
9	0.001256	0.117141	0.997942	0.965805	0.988247
10	0.000512	0.053978	0.979238	0.870985	0.935725
11	0.000157	0.004328	0.596811	0.333705	0.445643
12	0.000147	0.001103	0.315658	0.140354	0.206698
13	0.000146	0.000428	0.162166	0.062156	0.097445
14	0.000146	0.000401	0.152756	0.057916	0.091210
15	0.000146	0.000429	0.162354	0.062241	0.097570

	6	7	8	9	10
6	1.000000				
7	0.895637	1.000000			
8	0.999991	0.462063	1.000000		
9	0.999985	0.440164	1.000000	1.000000	
10	0.999130	0.258670	1.000000	1.000000	1.000000
11	0.843129	0.033367	0.996587	0.997437	0.999919
12	0.573694	0.009435	0.947023	0.954716	0.993121
13	0.352331	0.003296	0.815848	0.833050	0.949121
14	0.336341	0.003024	0.801038	0.818942	0.942309
15	0.352647	0.003301	0.816131	0.833320	0.949248

	11	12	13	14	15
11	1.000000				
12	1.000000	1.000000			
13	0.999979	1.000000	1.000000		
14	0.999968	1.000000	1.000000	1.000000	
15	0.999979	1.000000	1.000000	1.000000	1.000000

Appendix 27

Multiple comparisons of the mean values of ecdysteroid titres in male pharate-adults of *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

LEVELS ENCOUNTERED DURING PROCESSING ARE:

AGE	40	44	48	52	56	60	64	68	72	76	80
		84	88	92	96	100	104 h				

DEP VAR: TITRE N: 80 MULTIPLE R: 0.899 SQUARED MULTIPLE R: 0.809

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	1919966.296	16	127997.753	20.6608	0.000
ERROR	453558.873	68	7086.857		

COL/ROW	AGE
1	40
2	44
3	48
4	52
5	56
6	60
7	64
8	68
9	72
10	76
11	80
12	84
13	88
14	92
15	96
16	100
17	104

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRE

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	231.506000	0.000000			
3	277.045000	45.539000	0.000000		
4	335.261000	103.755000	58.216000	0.000000	
5	520.543600	289.037800	243.498800	185.282800	0.000000
6	331.293600	99.787600	54.248600	-3.967400	-189.250200
7	339.479800	107.973800	62.434800	4.218800	-181.064000
8	328.799800	97.293800	51.754800	-6.461200	-191.744000
9	200.878200	-30.627800	-76.166800	-134.382800	-319.665600
10	120.185000	-111.321000	-156.860000	-215.076000	-400.358800
11	78.024400	-153.481600	-199.020600	-257.236600	-442.519400
12	52.120600	-179.385400	-224.924400	-283.140400	-468.423200
13	-32.043560	-263.549560	-309.088560	-367.304560	-552.587360
14	337.429400	105.923400	60.384400	2.168400	-183.114400
15	225.682400	-5.823600	-51.362600	-109.578600	-294.861400
16	-17.410600	-248.916600	-294.455600	-352.671600	-537.954400
17	-45.376000	-276.882000	-322.421000	-380.637000	-565.919800

	6	7	8	9	10
6	0.000000				
7	8.186200	0.000000			
8	-2.493800	-10.680000	0.000000		
9	-130.415400	-138.601600	-127.921600	0.000000	
10	-211.108600	-219.294800	-208.614800	-80.693200	0.000000
11	-253.269200	-261.455400	-250.775400	-122.853800	-42.160600
12	-279.173000	-287.359200	-276.679200	-148.757600	-68.064400
13	-363.337160	-371.523360	-360.843360	-232.921760	-152.228560
14	6.135800	-2.050400	8.629600	136.551200	217.244400
15	-105.611200	-113.797400	-103.117400	24.804200	105.497400
16	-348.704200	-356.890400	-346.210400	-218.288800	-137.595600
17	-376.669600	-384.855800	-374.175800	-246.254200	-165.561000

	11	12	13	14	15
11	0.000000				
12	-25.903800	0.000000			
13	-110.067960	-84.164160	0.000000		
14	259.405000	285.308800	369.472960	0.000000	
15	147.658000	173.561800	257.725960	-111.747000	0.000000
16	-95.435000	-69.531200	14.632960	-354.840000	-243.093000
17	-123.400400	-97.496600	-13.332440	-382.805400	-271.058400

	16	17
16	0.000000	
17	-27.965400	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.003445	1.000000			
3	0.000294	0.999971	1.000000		
4	0.000168	0.838651	0.999330	1.000000	
5	0.000167	0.000213	0.001612	0.052864	1.000000
6	0.000169	0.875138	0.999717	1.000000	0.042767
7	0.000168	0.794297	0.998457	1.000000	0.065868
8	0.000169	0.895305	0.999844	1.000000	0.037339
9	0.022345	1.000000	0.986911	0.448323	0.000171
10	0.641350	0.755482	0.202312	0.009612	0.000167
11	0.983475	0.231800	0.024851	0.000714	0.000167
12	0.999829	0.071734	0.005219	0.000249	0.000167
13	1.000000	0.000510	0.000176	0.000167	0.000167
14	0.000168	0.816535	0.998967	1.000000	0.059262
15	0.004978	1.000000	0.999858	0.776055	0.000196
16	1.000000	0.001156	0.000197	0.000167	0.000167
17	0.999972	0.000296	0.000170	0.000167	0.000167

	6	7	8	9	10
6	1.000000				
7	1.000000	1.000000			
8	1.000000	1.000000	1.000000		
9	0.501356	0.394086	0.535374	1.000000	
10	0.012237	0.007403	0.014201	0.977344	1.000000
11	0.000893	0.000571	0.001034	0.605010	0.999990
12	0.000276	0.000220	0.000298	0.277842	0.995904
13	0.000167	0.000167	0.000167	0.003151	0.243458
14	1.000000	1.000000	1.000000	0.420171	0.008419
15	0.819810	0.725012	0.844868	1.000000	0.820996
16	0.000168	0.000167	0.000168	0.007894	0.406741
17	0.000167	0.000167	0.000167	0.001359	0.139101

	11	12	13	14	15
11	1.000000				
12	1.000000	1.000000			
13	0.770356	0.966929	1.000000		
14	0.000635	0.000230	0.000167	1.000000	
15	0.289310	0.095670	0.000695	0.750340	1.000000
16	0.908930	0.994848	1.000000	0.000167	0.001653
17	0.597517	0.893746	1.000000	0.000167	0.000364

	16	17
16	1.000000	
17	1.000000	1.000000

LEVELS ENCOUNTERED DURING PROCESSING ARE:
AGE

104	108	112	116	120	124
128	132	136	140	144	148
152	156	160	h		

DEP VAR: TITRE N: 75 MULTIPLE R: 0.952 SQUARED MULTIPLE R: 0.907

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	192499.486	14	13749.963	41.642	0.000
ERROR	19811.544	60	330.192		

COL/ROW	AGE
1	104
2	108
3	112
4	116
5	120
6	124
7	128
8	132
9	136
10	140
11	144
12	148
13	152
14	156
15	160

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRE

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	-13.679400	0.000000			
3	-61.771600	-48.092200	0.000000		
4	-68.796400	-55.117000	-7.024800	0.000000	
5	-54.609400	-40.930000	7.162200	14.187000	0.000000
6	-92.667800	-78.988400	-30.896200	-23.871400	-38.058400
7	-95.038000	-81.358600	-33.266400	-26.241600	-40.428600
8	-100.746600	-87.067200	-38.975000	-31.950200	-46.137200
9	-128.599600	-114.920200	-66.828000	-59.803200	-73.990200
10	-132.035200	-118.355800	-70.263600	-63.238800	-77.425800
11	-146.344000	-132.664600	-84.572400	-77.547600	-91.734600
12	-157.118600	-143.439200	-95.347000	-88.322200	-102.509200
13	-155.445400	-141.766000	-93.673800	-86.649000	-100.836000
14	-156.079600	-142.400200	-94.308000	-87.283200	-101.470200
15	-154.973600	-141.294200	-93.202000	-86.177200	-100.364200

	6	7	8	9	10
6	0.000000				
7	-2.370200	0.000000			
8	-8.078800	-5.708600	0.000000		
9	-35.931800	-33.561600	-27.853000	0.000000	
10	-39.367400	-36.997200	-31.288600	-3.435600	0.000000
11	-53.676200	-51.306000	-45.597400	-17.744400	-14.308800
12	-64.450800	-62.080600	-56.372000	-28.519000	-25.083400
13	-62.777600	-60.407400	-54.698800	-26.845800	-23.410200
14	-63.411800	-61.041600	-55.333000	-27.480000	-24.044400
15	-62.305800	-59.935600	-54.227000	-26.374000	-22.938400

	11	12	13	14	15
11	0.000000				
12	-10.774600	0.000000			
13	-9.101400	1.673200	0.000000		
14	-9.735600	1.039000	-0.634200	0.000000	
15	-8.629600	2.145000	0.471800	1.106000	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.996662	1.000000			
3	0.000254	0.007532	1.000000		
4	0.000154	0.001079	0.999999	1.000000	
5	0.001238	0.046779	0.999998	0.995180	1.000000
6	0.000145	0.000145	0.336851	0.743976	0.089448
7	0.000145	0.000145	0.228691	0.606105	0.052646
8	0.000145	0.000145	0.073207	0.285564	0.012717
9	0.000145	0.000145	0.000163	0.000353	0.000146
10	0.000145	0.000145	0.000150	0.000208	0.000146
11	0.000145	0.000145	0.000145	0.000146	0.000145
12	0.000145	0.000145	0.000145	0.000145	0.000145
13	0.000145	0.000145	0.000145	0.000145	0.000145
14	0.000145	0.000145	0.000145	0.000145	0.000145
15	0.000145	0.000145	0.000145	0.000145	0.000145

	6	7	8	9	10
6	1.000000				
7	1.000000	1.000000			
8	0.999992	1.000000	1.000000		
9	0.138998	0.217095	0.508272	1.000000	
10	0.067070	0.111929	0.317176	1.000000	1.000000
11	0.001598	0.003101	0.014646	0.963882	0.994759
12	0.000186	0.000244	0.000777	0.468583	0.675345
13	0.000224	0.000316	0.001208	0.569361	0.768526
14	0.000205	0.000284	0.001019	0.530797	0.734504
15	0.000237	0.000345	0.001373	0.598070	0.792556

	11	12	13	14	15
11	1.000000				
12	0.999752	1.000000			
13	0.999966	1.000000	1.000000		
14	0.999924	1.000000	1.000000	1.000000	
15	0.999982	1.000000	1.000000	1.000000	1.000000

Appendix 28

Comparison between ecdysteroid titres during pharate-adult stage in the females and males of *Lucilia cuprina* using t-Test.

	Female	Male
Mean	302.15	278.35
Standard Error	18.85	17.39
Median	216.86	213.98
Standard Deviation	234.65	216.44
Variance	55059.8	46847.07
Kurtosis	-1.06	-0.7
Skewness	0.59	0.69
Range	817.2	806.75
Minimum	30.97	25.94
Maximum	848.18	832.69
Sum	46833.2	43144.72
Count	155	155

t-Test: Paired Two-Sample for Means

	Female	Male
Mean	302.15	278.35
Variance	55059.8	46847.07
Observations	155	155
Pearson Correlation	0.91	
Pooled Variance	46273.23	
Hypothesized Mean Difference	0	
df	154	
t	3.06	
P(T<=t) one-tail	0	
t Critical one-tail	1.65	
P(T<=t) two-tail	0	
t Critical two-tail	1.98	

Appendix 29

Comparison between female and male ecdysteroid peaks during pharate-adult stage of *Lucilia cuprina* at 4 h using t-Test.

(68 h Female)
(56 h Male)

t-Test: Two-Sample for Means

	Female	Male
Mean	710.46	764.37
Variance	9958.53	3212.86
Observations	5	5
Pearson Correlation	0.41	
Pooled Variance	2327.93	
Hypothesized Mean Difference	0	
df	4	
t	1.31	
P(T<=t) one-tail	0.13	
t Critical one-tail	2.13	
P(T<=t) two-tail	0.26	
t Critical two-tail	2.78	

92 *h*

t-Test: Two-Sample for Means

	Female	Male
Mean	646.79	581.26
Variance	2117.24	13490.81
Observations	5	5
Pearson Correlation	0.45	
Pooled Variance	2384.79	
Hypothesized Mean Difference	0	
df	4	
t	1.41	
P(T<=t) one-tail	0.12	
t Critical one-tail	2.13	
P(T<=t) two-tail	0.23	
t Critical two-tail	2.78	

Appendix 30

Multiple comparisons of mean body weights of non liver-fed adult females *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

```
LEVELS ENCOUNTERED DURING PROCESSING ARE:
AGE    0       4       8       12      16      20      24      28      32      36      40
       44      48      h
```

DEP VAR: WEIGHT N:65 MULTIPLE R: 0.661 SQUARED MULTIPLE R: 0.437

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	592.620377	12	49.385031	3.365727	0.001131
ERROR	762.991663	52	14.672917		

COL/ ROW	AGE
1	0
2	4
3	8
4	12
5	16
6	20
7	24
8	28
9	32
10	36
11	40
12	44
13	48

USING LEAST SQUARES MEANS.
POST HOC TEST OF WEIGHT

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	-1.554000	0.000000			
3	-7.388000	-5.834000	0.000000		
4	-2.492000	-0.938000	4.896000	0.000000	
5	-0.258000	1.296000	7.130000	2.234000	0.000000
6	-2.534000	-0.980000	4.854000	-0.042000	-2.276000
7	-0.202000	1.352000	7.186000	2.290000	0.056000
8	-0.442000	1.112000	6.946000	2.050000	-0.184000
9	-0.948000	0.606000	6.440000	1.544000	-0.690000
10	5.933000	7.487000	13.321000	8.425000	6.191000
11	2.546333	4.100333	9.934333	5.038333	2.804333
12	-0.742000	0.812000	6.646000	1.750000	-0.484000
13	3.636000	5.190000	11.024000	6.128000	3.894000

	6	7	8	9	10
6	0.000000				
7	2.332000	0.000000			
8	2.092000	-0.240000	0.000000		
9	1.586000	-0.746000	-0.506000	0.000000	
10	8.467000	6.135000	6.375000	6.881000	0.000000
11	5.080333	2.748333	2.988333	3.494333	-3.386667
12	1.792000	-0.540000	-0.300000	0.206000	-6.675000
13	6.170000	3.838000	4.078000	4.584000	-2.297000

	11	12	13
11	0.000000		
12	-3.288333	0.000000	
13	1.089667	4.378000	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.999983	1.000000			
3	0.138777	0.454860	1.000000		
4	0.997843	1.000000	0.714818	1.000000	
5	1.000000	0.999998	0.174712	0.999255	1.000000
6	0.997467	1.000000	0.725772	1.000000	0.999107
7	1.000000	0.999996	0.166364	0.999052	1.000000
8	1.000000	1.000000	0.204369	0.999684	1.000000
9	1.000000	1.000000	0.303995	0.999984	1.000000
10	0.521207	0.185777	0.000349	0.081289	0.454067
11	0.996020	0.856152	0.004917	0.614652	0.990690
12	1.000000	1.000000	0.260309	0.999939	1.000000
13	0.949509	0.634921	0.002144	0.377931	0.919608

	6	7	8	9	10
6	1.000000				
7	0.998860	1.000000			
8	0.999612	1.000000	1.000000		
9	0.999979	1.000000	1.000000	1.000000	
10	0.078104	0.468421	0.407950	0.293552	1.000000
11	0.602307	0.992169	0.984180	0.948154	0.974309
12	0.999921	1.000000	1.000000	1.000000	0.337667
13	0.367473	0.926907	0.892366	0.792195	0.999452

	11	12	13
11	1.000000		
12	0.966629	1.000000	
13	0.999999	0.837227	1.000000

LEVELS ENCOUNTERED DURING PROCESSING ARE:
AGE

48	52	56	60	64	68	72	76	80	84	88
92	96	100	104	108	112	116	h			

DEP VAR: WEIGHT N: 90 MULTIPLE R: 0.550 SQUARED MULTIPLE R: 0.302

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	237.124277	17	13.948487	1.835261	0.039580
ERROR	547.219653	72	7.600273		

COL/ ROW	AGE
1	48
2	52
3	56
4	60
5	64
6	68
7	72
8	76
9	80
10	84
11	88
12	92
13	96
14	100
15	104
16	108
17	112
18	116

USING LEAST SQUARES MEANS.
POST HOC TEST OF WEIGHT

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	-1.034000	0.000000			
3	3.212000	4.246000	0.000000		
4	3.080000	4.114000	-0.132000	0.000000	
5	1.150000	2.184000	-2.062000	-1.930000	0.000000
6	1.078000	2.112000	-2.134000	-2.002000	-0.072000
7	1.058000	2.092000	-2.154000	-2.022000	-0.092000
8	2.642000	3.676000	-0.570000	-0.438000	1.492000
9	5.352800	6.386800	2.140800	2.272800	4.202800
10	-1.356000	-0.322000	-4.568000	-4.436000	-2.506000
11	-0.788000	0.246000	-4.000000	-3.868000	-1.938000
12	0.146000	1.180000	-3.066000	-2.934000	-1.004000
13	1.152000	2.186000	-2.060000	-1.928000	0.002000
14	0.130000	1.164000	-3.082000	-2.950000	-1.020000
15	0.476000	1.510000	-2.736000	-2.604000	-0.674000
16	0.094000	1.128000	-3.118000	-2.986000	-1.056000
17	0.796000	1.830000	-2.416000	-2.284000	-0.354000
18	0.972000	2.006000	-2.240000	-2.108000	-0.178000

	6	7	8	9	10
6	0.000000				
7	-0.020000	0.000000			
8	1.564000	1.584000	0.000000		
9	4.274800	4.294800	2.710800	0.000000	
10	-2.434000	-2.414000	-3.998000	-6.708800	0.000000
11	-1.866000	-1.846000	-3.430000	-6.140800	0.568000
12	-0.932000	-0.912000	-2.496000	-5.206800	1.502000
13	0.074000	0.094000	-1.490000	-4.200800	2.508000
14	-0.948000	-0.928000	-2.512000	-5.222800	1.486000
15	-0.602000	-0.582000	-2.166000	-4.876800	1.832000
16	-0.984000	-0.964000	-2.548000	-5.258800	1.450000
17	-0.282000	-0.262000	-1.846000	-4.556800	2.152000
18	-0.106000	-0.086000	-1.670000	-4.380800	2.328000

	11	12	13	14	15
11	0.000000				
12	0.934000	0.000000			
13	1.940000	1.006000	0.000000		
14	0.918000	-0.016000	-1.022000	0.000000	
15	1.264000	0.330000	-0.676000	0.346000	0.000000
16	0.882000	-0.052000	-1.058000	-0.036000	-0.382000
17	1.584000	0.650000	-0.356000	0.666000	0.320000
18	1.760000	0.826000	-0.180000	0.842000	0.496000

	16	17	18
16	0.000000		
17	0.702000	0.000000	
18	0.878000	0.176000	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	1.000000	1.000000			
3	0.922865	0.584806	1.000000		
4	0.945078	0.638899	1.000000	1.000000	
5	1.000000	0.998399	0.999214	0.999659	1.000000
6	1.000000	0.998945	0.998795	0.999456	1.000000
7	1.000000	0.998061	0.998643	0.999384	1.000000
8	0.986698	0.802459	1.000000	1.000000	0.999990
9	0.196588	0.044193	0.998747	0.997433	0.602595
10	0.999998	1.000000	0.453520	0.506631	0.992316
11	1.000000	1.000000	0.684511	0.735102	0.999640
12	1.000000	1.000000	0.947132	0.963888	1.000000
13	1.000000	0.998382	0.999223	0.999663	1.000000
14	1.000000	1.000000	0.944780	0.962101	1.000000
15	1.000000	0.999988	0.981182	0.988518	1.000000
16	1.000000	1.000000	0.939210	0.957841	1.000000
17	1.000000	0.999829	0.994835	0.997284	1.000000
18	1.000000	0.999442	0.997837	0.998969	1.000000

	6	7	8	9	10
6	1.000000				
7	1.000000	1.000000			
8	0.999980	0.999976	1.000000		
9	0.572921	0.564667	0.982811	1.000000	
10	0.994397	0.994882	0.685298	0.025847	1.000000
11	0.999780	0.999809	0.874483	0.065261	1.000000
12	1.000000	1.000000	0.992642	0.234662	0.999989
13	1.000000	1.000000	0.999990	0.603417	0.992250
14	1.000000	1.000000	0.992118	0.230258	0.999990
15	1.000000	1.000000	0.998550	0.338012	0.999827
16	1.000000	1.000000	0.990844	0.220556	0.999993
17	1.000000	1.000000	0.999809	0.457963	0.998659
18	1.000000	1.000000	0.999951	0.529227	0.996602

	11	12	13	14	15
11	1.000000				
12	1.000000	1.000000			
13	0.999635	1.000000	1.000000		
14	1.000000	1.000000	1.000000	1.000000	
15	0.999999	1.000000	1.000000	1.000000	1.000000
16	1.000000	1.000000	1.000000	1.000000	1.000000
17	0.999976	1.000000	1.000000	1.000000	1.000000
18	0.999899	1.000000	1.000000	1.000000	1.000000

	16	17	18
16	1.000000		
17	1.000000	1.000000	
18	1.000000	1.000000	1.000000

Appendix 31

Multiple comparisons of mean body weights of *ad libitum* liver-fed adult females *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

LEVELS ENCOUNTERED DURING PROCESSING ARE:

AGE	0	4	8	12	16	20	24	28	32	36	40
	44	48	h							

DEP VAR: WEIGHT N: 65 MULTIPLE R: 0.929 SQUARED MULTIPLE R: 0.864

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	3446.208068	12	287.184006	27.452662	0.000000
ERROR	543.975230	52	10.461062		

COL/ROW	AGE (h)
1	0
2	4
3	8
4	12
5	16
6	20
7	24
8	28
9	32
10	36
11	40
12	44
13	48

USING LEAST SQUARES MEANS. POST HOC TEST OF WEIGHT

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	-0.506000	0.000000			
3	-1.248000	-0.742000	0.000000		
4	2.524000	3.030000	3.772000	0.000000	
5	1.296000	1.802000	2.544000	-1.228000	0.000000
6	-0.474000	0.032000	0.774000	-2.998000	-1.770000
7	3.064000	3.570000	4.312000	0.540000	1.768000
8	15.194000	15.700000	16.442000	12.670000	13.898000
9	15.716000	16.222000	16.964000	13.192000	14.420000
10	15.158000	15.664000	16.406000	12.634000	13.862000
11	15.653000	16.159000	16.901000	13.129000	14.357000
12	12.290000	12.796000	13.538000	9.766000	10.994000
13	15.876000	16.382000	17.124000	13.352000	14.580000

	6	7	8	9	10
6	0.000000				
7	3.538000	0.000000			
8	15.668000	12.130000	0.000000		
9	16.190000	12.652000	0.522000	0.000000	
10	15.632000	12.094000	-0.036000	-0.558000	0.000000
11	16.127000	12.589000	0.459000	-0.063000	0.495000
12	12.764000	9.226000	-2.904000	-3.426000	-2.868000
13	16.350000	12.812000	0.682000	0.160000	0.718000

	11	12	13
11	0.000000		
12	-3.363000	0.000000	
13	0.223000	3.586000	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	1.000000	1.000000			
3	0.999990	1.000000	1.000000		
4	0.988938	0.954015	0.818388	1.000000	
5	0.999985	0.999526	0.988179	0.999992	1.000000
6	1.000000	1.000000	1.000000	0.957392	0.999604
7	0.950222	0.866411	0.657996	1.000000	0.999609
8	0.000129	0.000129	0.000129	0.000133	0.000129
9	0.000129	0.000129	0.000129	0.000130	0.000129
10	0.000129	0.000129	0.000129	0.000148	0.000131
11	0.000129	0.000129	0.000129	0.000129	0.000129
12	0.000138	0.000132	0.000130	0.001088	0.000237
13	0.000129	0.000129	0.000129	0.000130	0.000129

	6	7	8	9	10
6	1.000000				
7	0.873290	1.000000			
8	0.000129	0.000141	1.000000		
9	0.000129	0.000133	1.000000	1.000000	
10	0.000129	0.000180	1.000000	1.000000	1.000000
11	0.000129	0.000130	1.000000	1.000000	1.000000
12	0.000133	0.002425	0.966285	0.895653	0.980547
13	0.000129	0.000132	1.000000	1.000000	1.000000

	11	12	13
11	1.000000		
12	0.878605	1.000000	
13	1.000000	0.862901	1.000000

LEVELS ENCOUNTERED DURING PROCESSING ARE:

AGE	48	52	56	60	64	68	72	76	80	84	88
	92	96	100	104	108	112	116	h			

DEP VAR: WEIGHT N: 90 MULTIPLE R: 0.418 SQUARED MULTIPLE R: 0.175

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	220.342000	17	12.961294	0.896356	0.580345
ERROR	1041.119440	72	14.459992		

COL/ ROW	AGE (h)
1	48
2	52
3	56
4	60
5	64
6	68
7	72
8	76
9	80
10	84
11	88
12	92
13	96
14	100
15	104
16	108
17	112
18	116

USING LEAST SQUARES MEANS. POST HOC TEST OF WEIGHT

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	2.656000	0.000000			
3	1.582000	-1.074000	0.000000		
4	-1.910000	-4.566000	-3.492000	0.000000	
5	0.560000	-2.096000	-1.022000	2.470000	0.000000
6	3.410000	0.754000	1.828000	5.320000	2.850000
7	2.030000	-0.626000	0.448000	3.940000	1.470000
8	-1.832000	-4.488000	-3.414000	0.078000	-2.392000
9	1.100000	-1.556000	-0.482000	3.010000	0.540000
10	2.134000	-0.522000	0.552000	4.044000	1.574000
11	2.246000	-0.410000	0.664000	4.156000	1.686000
12	-0.348000	-3.004000	-1.930000	1.562000	-0.908000
13	1.118000	-1.538000	-0.464000	3.028000	0.558000
14	1.034000	-1.622000	-0.548000	2.944000	0.474000
15	2.126000	-0.530000	0.544000	4.036000	1.566000
16	1.514000	-1.142000	-0.068000	3.424000	0.954000
17	1.442000	-1.214000	-0.140000	3.352000	0.882000
18	-2.182000	-4.838000	-3.764000	-0.272000	-2.742000

	6	7	8	9	10
6	0.000000				
7	-1.380000	0.000000			
8	-5.242000	-3.862000	0.000000		
9	-2.310000	-0.930000	2.932000	0.000000	
10	-1.276000	0.104000	3.966000	1.034000	0.000000
11	-1.164000	0.216000	4.078000	1.146000	0.112000
12	-3.758000	-2.378000	1.484000	-1.448000	-2.482000
13	-2.292000	-0.912000	2.950000	0.018000	-1.016000
14	-2.376000	-0.996000	2.866000	-0.066000	-1.100000
15	-1.284000	0.096000	3.958000	1.026000	-0.008000
16	-1.896000	-0.516000	3.346000	0.414000	-0.620000
17	-1.968000	-0.588000	3.274000	0.342000	-0.692000
18	-5.592000	-4.212000	-0.350000	-3.282000	-4.316000

	11	12	13	14	15
11	0.000000				
12	-2.594000	0.000000			
13	-1.128000	1.466000	0.000000		
14	-1.212000	1.382000	-0.084000	0.000000	
15	-0.120000	2.474000	1.008000	1.092000	0.000000
16	-0.732000	1.862000	0.396000	0.480000	-0.612000
17	-0.804000	1.790000	0.324000	0.408000	-0.684000
18	-4.428000	-1.834000	-3.300000	-3.216000	-4.308000

	16	17	18
16	0.000000		
17	-0.072000	0.000000	
18	-3.696000	-3.624000	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.999669	1.000000			
3	1.000000	1.000000	1.000000		
4	0.999997	0.902891	0.991442	1.000000	
5	1.000000	0.999987	1.000000	0.999872	1.000000
6	0.993364	1.000000	0.999998	0.739204	0.999194
7	0.999992	1.000000	1.000000	0.971680	1.000000
8	0.999998	0.914747	0.993280	1.000000	0.999917
9	1.000000	1.000000	1.000000	0.998415	1.000000
10	0.999983	1.000000	1.000000	0.964124	1.000000
11	0.999965	1.000000	1.000000	0.954412	0.999999
12	1.000000	0.998452	0.999996	1.000000	1.000000
13	1.000000	1.000000	1.000000	0.998298	1.000000
14	1.000000	1.000000	1.000000	0.998792	1.000000
15	0.999984	1.000000	1.000000	0.964753	1.000000
16	1.000000	1.000000	1.000000	0.993065	1.000000
17	1.000000	1.000000	1.000000	0.994493	1.000000
18	0.999977	0.853714	0.981655	1.000000	0.999502

	6	7	8	9	10
6	1.000000				
7	1.000000	1.000000			
8	0.759775	0.976514	1.000000		
9	0.999949	1.000000	0.998850	1.000000	
10	1.000000	1.000000	0.969915	1.000000	1.000000
11	1.000000	1.000000	0.961356	1.000000	1.000000
12	0.981937	0.999923	1.000000	1.000000	0.999864
13	0.999954	1.000000	0.998762	1.000000	1.000000
14	0.999924	1.000000	0.999136	1.000000	1.000000
15	1.000000	1.000000	0.970467	1.000000	1.000000
16	0.999997	1.000000	0.994600	1.000000	1.000000
17	0.999995	1.000000	0.995760	1.000000	1.000000
18	0.663030	0.948898	1.000000	0.995637	0.937428

	11	12	13	14	15
11	1.000000				
12	0.999756	1.000000			
13	1.000000	1.000000	1.000000		
14	1.000000	1.000000	1.000000	1.000000	
15	1.000000	0.999869	1.000000	1.000000	1.000000
16	1.000000	0.999998	1.000000	1.000000	1.000000
17	1.000000	0.999999	1.000000	1.000000	1.000000
18	0.923194	0.999998	0.995364	0.996542	0.938368

	16	17	18
16	1.000000		
17	1.000000	1.000000	
18	0.984674	0.987425	1.000000

Appendix 32

Comparison of body weights between *ad lib.* liver-fed and non liver-fed female adults of *Lucilia cuprina* using t-Test

	Liver-fed female	Non liver-fed female
Mean	46.85	38.63
Standard Error	0.61	0.34
Median	49.11	38.72
Mode	49.09	39.99
Standard Deviation	7.43	4.2
Variance	55.22	17.6
Kurtosis	-0.44	-0.28
Skewness	-0.75	0.07
Range	32.1	19.65
Minimum	29.59	29.48
Maximum	61.69	49.13
Sum	7026.87	5794.44
Count	150	150

t-Test: Paired Two-Sample for Means

	Liver-fed female	Non liver-fed female
Mean	46.85	38.63
Variance	55.22	17.6
Observations	150	150
Pearson Correlation	0.51	
Pooled Variance	15.94	
Hypothesized Mean Difference	.0	
df	149	
t	15.73	
P(T<=t) one-tail	0.000000	
t Critical one-tail	1.66	
P(T<=t) two-tail	0.000000	
t Critical two-tail	1.98	

Appendix 33

Multiple comparisons of mean weights of female reproductive system of *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

LEVELS ENCOUNTERED DURING PROCESSING ARE:

AGE	48	52	56	60	64	68	72	76	80	84	88
	92	96	h							

DEP VAR: WEIGHT N: 65 MULTIPLE R: 0.935 SQUARED MULTIPLE R: 0.875

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	1215.277818	12	101.273152	30.279990	0.000000
ERROR	173.916960	52	3.344557		

COL/ ROW	AGE (h)
1	48
2	52
3	56
4	60
5	64
6	68
7	72
8	76
9	80
10	84
11	88
12	92
13	96

USING LEAST SQUARES MEANS. POST HOC TEST OF WEIGHT

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	0.358000	0.000000			
3	0.902000	0.544000	0.000000		
4	1.070000	0.712000	0.168000	0.000000	
5	1.904000	1.546000	1.002000	0.834000	0.000000
6	2.984000	2.626000	2.082000	1.914000	1.080000
7	3.152000	2.794000	2.250000	2.082000	1.248000
8	6.442000	6.084000	5.540000	5.372000	4.538000
9	7.942000	7.584000	7.040000	6.872000	6.038000
10	10.722000	10.364000	9.820000	9.652000	8.818000
11	10.610000	10.252000	9.708000	9.540000	8.706000
12	10.948000	10.590000	10.046000	9.878000	9.044000
13	11.136000	10.778000	10.234000	10.066000	9.232000

	6	7	8	9	10
6	0.000000				
7	0.168000	0.000000			
8	3.458000	3.290000	0.000000		
9	4.958000	4.790000	1.500000	0.000000	
10	7.738000	7.570000	4.280000	2.780000	0.000000
11	7.626000	7.458000	4.168000	2.668000	-0.112000
12	7.964000	7.796000	4.506000	3.006000	0.226000
13	8.152000	7.984000	4.694000	3.194000	0.414000

	11	12	13
11	0.000000		
12	0.338000	0.000000	
13	0.526000	0.188000	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	1.000000	1.000000			
3	0.999863	0.999999	1.000000		
4	0.999232	0.999989	1.000000	1.000000	
5	0.906321	0.978761	0.999599	0.999940	1.000000
6	0.347948	0.547499	0.840727	0.903178	0.999158
7	0.269280	0.449958	0.761458	0.840727	0.996611
8	0.000181	0.000295	0.001039	0.001604	0.014347
9	0.000129	0.000130	0.000136	0.000141	0.000322
10	0.000129	0.000129	0.000129	0.000129	0.000129
11	0.000129	0.000129	0.000129	0.000129	0.000129
12	0.000129	0.000129	0.000129	0.000129	0.000129
13	0.000129	0.000129	0.000129	0.000129	0.000129

	6	7	8	9	10
6	1.000000				
7	1.000000	1.000000			
8	0.158195	0.213900	1.000000		
9	0.004864	0.007537	0.983315	1.000000	
10	0.000129	0.000130	0.027008	0.457887	1.000000
11	0.000130	0.000130	0.035199	0.522735	1.000000
12	0.000129	0.000129	0.015541	0.336972	1.000000
13	0.000129	0.000129	0.009651	0.251455	1.000000

	11	12	13
11	1.000000		
12	1.000000	1.000000	
13	1.000000	1.000000	1.000000

Appendix 34

Multiple comparisons of mean body weight of female *Lucilia cuprina* minus reproductive system, using one-way analysis of variance and the Tukey test of hypothesis.

LEVELS ENCOUNTERED DURING PROCESSING ARE:
AGE

48	52	56	60	64	68	72	76	80	84	88
92	96	h								

DEP VAR: WEIGHT N: 65 MULTIPLE R: 0.742 SQUARED MULTIPLE R: 0.551

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	1361.281255	12	113.440105	5.310950	0.000009
ERROR	1110.702600	52	21.359665		

COL/ ROW	AGE (h)
1	48
2	52
3	56
4	60
5	64
6	68
7	72
8	76
9	80
10	84
11	88
12	92
13	96

USING LEAST SQUARES MEANS. POST HOC TEST OF WEIGHT

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	-0.356000	0.000000			
3	-0.738000	-0.382000	0.000000		
4	-4.490000	-4.134000	-3.752000	0.000000	
5	-4.312000	-3.956000	-3.574000	0.178000	0.000000
6	-3.170000	-2.814000	-2.432000	1.320000	1.142000
7	-6.616000	-6.260000	-5.878000	-2.126000	-2.304000
8	-10.944000	-10.588000	-10.206000	-6.454000	-6.632000
9	-9.952000	-9.596000	-9.214000	-5.462000	-5.640000
10	-11.498000	-11.142000	-10.760000	-7.008000	-7.186000
11	-10.694000	-10.338000	-9.956000	-6.204000	-6.382000
12	-12.836000	-12.480000	-12.098000	-8.346000	-8.524000
13	-11.528000	-11.172000	-10.790000	-7.038000	-7.216000

	6	7	8	9	10
6	0.000000				
7	-3.446000	0.000000			
8	-7.774000	-4.328000	0.000000		
9	-6.782000	-3.336000	0.992000	0.000000	
10	-8.328000	-4.882000	-0.554000	-1.546000	0.000000
11	-7.524000	-4.078000	0.250000	-0.742000	0.804000
12	-9.666000	-6.220000	-1.892000	-2.884000	-1.338000
13	-8.358000	-4.912000	-0.584000	-1.576000	-0.030000

	11	12	13
11	0.000000		
12	-2.142000	0.000000	
13	-0.834000	1.308000	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	1.000000	1.000000			
3	1.000000	1.000000	1.000000		
4	0.940648	0.967220	0.984646	1.000000	
5	0.955339	0.976581	0.989759	1.000000	1.000000
6	0.996436	0.998858	0.999734	1.000000	1.000000
7	0.552236	0.635361	0.721141	0.999934	0.999847
8	0.023914	0.033451	0.047457	0.590183	0.548493
9	0.059385	0.080458	0.109858	0.805193	0.770758
10	0.013925	0.019763	0.028478	0.461812	0.422307
11	0.030301	0.042084	0.059179	0.648264	0.607010
12	0.003525	0.005120	0.007591	0.209299	0.184815
13	0.013516	0.019196	0.027684	0.455064	0.415786

	6	7	8	9	10
6	1.000000				
7	0.992503	1.000000			
8	0.303271	0.954139	1.000000		
9	0.513540	0.994365	1.000000	1.000000	
10	0.211900	0.897418	1.000000	0.999998	1.000000
11	0.351334	0.970420	1.000000	1.000000	1.000000
12	0.075869	0.644586	0.999981	0.998544	1.000000
13	0.207578	0.893468	1.000000	0.999997	1.000000

	11	12	13
11	1.000000		
12	0.999929	1.000000	
13	1.000000	1.000000	1.000000

Appendix 35

Multiple comparisons of mean body weights of liver-fed adult males *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

```
LEVELS ENCOUNTERED DURING PROCESSING ARE:
AGE
       0       4       8       12      16      20      24      28      32      36      40
       44      48      h
```

DEP VAR: WEIGHT N: 65 MULTIPLE R: 0.759 SQUARED MULTIPLE R: 0.577

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	547.191168	12	45.599264	5.906808	0.000002
ERROR	401.428638	52	7.719782		

COL/ ROW	AGE
1	0
2	4
3	8
4	12
5	16
6	20
7	24
8	28
9	32
10	36
11	40
12	44
13	48

USING LEAST SQUARES MEANS. POST HOC TEST OF WEIGHT

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	-7.048000	0.000000			
3	-3.864000	3.184000	0.000000		
4	-3.806000	3.242000	0.058000	0.000000	
5	-3.232000	3.816000	0.632000	0.574000	0.000000
6	-5.316000	1.732000	-1.452000	-1.510000	-2.084000
7	-1.846000	5.202000	2.018000	1.960000	1.386000
8	1.712000	8.760000	5.576000	5.518000	4.944000
9	2.050000	9.098000	5.914000	5.856000	5.282000
10	1.571500	8.619500	5.435500	5.377500	4.803500
11	1.672333	8.720333	5.536333	5.478333	4.904333
12	-2.856000	4.192000	1.008000	0.950000	0.376000
13	-3.868000	3.180000	-0.004000	-0.062000	-0.636000

	6	7	8	9	10
6	0.000000				
7	3.470000	0.000000			
8	7.028000	3.558000	0.000000		
9	7.366000	3.896000	0.338000	0.000000	
10	6.887500	3.417500	-0.140500	-0.478500	0.000000
11	6.988333	3.518333	-0.039667	-0.377667	0.100833
12	2.460000	-1.010000	-4.568000	-4.906000	-4.427500
13	1.448000	-2.022000	-5.580000	-5.918000	-5.439500

	11	12	13
11	0.000000		
12	-4.528333	0.000000	
13	-5.540333	-1.012000	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.011107	1.000000			
3	0.596417	0.834832	1.000000		
4	0.618922	0.817881	1.000000	1.000000	
5	0.820864	0.615051	1.000000	1.000000	1.000000
6	0.146447	0.998559	0.999752	0.999631	0.992109
7	0.997363	0.168456	0.994049	0.995409	0.999846
8	0.998710	0.000588	0.104635	0.112983	0.227410
9	0.993156	0.000354	0.065578	0.071214	0.152762
10	0.999695	0.001704	0.184778	0.197009	0.349507
11	0.998438	0.000350	0.078952	0.085861	0.185265
12	0.913633	0.469739	0.999995	0.999997	1.000000
13	0.594861	0.835969	1.000000	1.000000	1.000000

	6	7	8	9	10
6	1.000000				
7	0.743701	1.000000			
8	0.011485	0.712386	1.000000		
9	0.006475	0.583961	1.000000	1.000000	
10	0.027387	0.823807	1.000000	1.000000	1.000000
11	0.007262	0.669133	1.000000	1.000000	1.000000
12	0.969646	0.999995	0.336617	0.237202	0.476430
13	0.999759	0.999945	0.104078	0.065204	0.183955

	11	12	13
11	1.000000		
12	0.286394	1.000000	
13	0.078494	0.999995	1.000000

LEVELS ENCOUNTERED DURING PROCESSING ARE:
AGE

48 52 56 60 64 68 72 76 80 84 88

92 96 100 104 108 112 116 h

DEP VAR: WEIGHT N: 90 MULTIPLE R: 0.613 SQUARED MULTIPLE R: 0.376

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	241.071316	17	14.180666	2.548118	0.003124
ERROR	400.691040	72	5.565153		

COL/ ROW	AGE
1	48
2	52
3	56
4	60
5	64
6	68
7	72
8	76
9	80
10	84
11	88
12	92
13	96
14	100
15	104
16	108
17	112
18	116

USING LEAST SQUARES MEANS.
POST HOC TEST OF WEIGHT

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	1.486000	0.000000			
3	0.920000	-0.566000	0.000000		
4	2.316000	0.830000	1.396000	0.000000	
5	0.958000	-0.528000	0.038000	-1.358000	0.000000
6	-2.174000	-3.660000	-3.094000	-4.490000	-3.132000
7	-2.122000	-3.608000	-3.042000	-4.438000	-3.080000
8	-0.800000	-2.286000	-1.720000	-3.116000	-1.758000
9	1.328000	-0.158000	0.408000	-0.988000	0.370000
10	1.018000	-0.468000	0.098000	-1.298000	0.060000
11	-0.134000	-1.620000	-1.054000	-2.450000	-1.092000
12	2.458000	0.972000	1.538000	0.142000	1.500000
13	-1.450000	-2.936000	-2.370000	-3.766000	-2.408000
14	-3.538000	-5.024000	-4.458000	-5.854000	-4.496000
15	-1.100000	-2.586000	-2.020000	-3.416000	-2.058000
16	0.626000	-0.860000	-0.294000	-1.690000	-0.332000
17	0.956000	-0.530000	0.036000	-1.360000	-0.002000
18	-1.752000	-3.238000	-2.672000	-4.068000	-2.710000

	6	7	8	9	10
6	0.000000				
7	0.052000	0.000000			
8	1.374000	1.322000	0.000000		
9	3.502000	3.450000	2.128000	0.000000	
10	3.192000	3.140000	1.818000	-0.310000	0.000000
11	2.040000	1.988000	0.666000	-1.462000	-1.152000
12	4.632000	4.580000	3.258000	1.130000	1.440000
13	0.724000	0.672000	-0.650000	-2.778000	-2.468000
14	-1.364000	-1.416000	-2.738000	-4.866000	-4.556000
15	1.074000	1.022000	-0.300000	-2.428000	-2.118000
16	2.800000	2.748000	1.426000	-0.702000	-0.392000
17	3.130000	3.078000	1.756000	-0.372000	-0.062000
18	0.422000	0.370000	-0.952000	-3.080000	-2.770000

	11	12	13	14	15
11	0.000000				
12	2.592000	0.000000			
13	-1.316000	-3.908000	0.000000		
14	-3.404000	-5.996000	-2.088000	0.000000	
15	-0.966000	-3.558000	0.350000	2.438000	0.000000
16	0.760000	-1.832000	2.076000	4.164000	1.726000
17	1.090000	-1.502000	2.406000	4.494000	2.056000
18	-1.618000	-4.210000	-0.302000	1.786000	-0.652000

	16	17	18
16	0.000000		
17	0.330000	0.000000	
18	-2.378000	-2.708000	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.999915	1.000000			
3	1.000000	1.000000	1.000000		
4	0.983074	1.000000	0.999964	1.000000	
5	1.000000	1.000000	1.000000	0.999976	1.000000
6	0.991119	0.571940	0.821712	0.223667	0.807620
7	0.993141	0.596998	0.840099	0.240369	0.826766
8	1.000000	0.985128	0.999428	0.813618	0.999249
9	0.999982	1.000000	1.000000	1.000000	1.000000
10	1.000000	1.000000	1.000000	0.999987	1.000000
11	1.000000	0.999734	0.999999	0.971063	0.999999
12	0.970184	1.000000	0.999866	1.000000	0.999904
13	0.999940	0.874204	0.978829	0.520919	0.975371
14	0.630519	0.097655	0.233849	0.020566	0.221793
15	0.999999	0.953223	0.996012	0.687642	0.995087
16	1.000000	1.000000	1.000000	0.999542	1.000000
17	1.000000	1.000000	1.000000	0.999975	1.000000
18	0.999280	0.765604	0.938477	0.382063	0.931020

	6	7	8	9	10
6	1.000000				
7	1.000000	1.000000			
8	0.999971	0.999983	1.000000		
9	0.647589	0.671949	0.992930	1.000000	
10	0.784304	0.804586	0.998858	1.000000	1.000000
11	0.995542	0.996680	1.000000	0.999932	0.999998
12	0.182249	0.196707	0.757265	0.999998	0.999945
13	1.000000	1.000000	1.000000	0.916204	0.969057
14	0.999974	0.999956	0.925153	0.126806	0.203655
15	0.999999	1.000000	1.000000	0.973386	0.993278
16	0.910995	0.922980	0.999952	1.000000	1.000000
17	0.808375	0.827482	0.999259	1.000000	1.000000
18	1.000000	1.000000	1.000000	0.826766	0.918051

	11	12	13	14	15
11	1.000000				
12	0.952286	1.000000			
13	0.999984	0.453920	1.000000		
14	0.693133	0.015347	0.994243	1.000000	
15	1.000000	0.620977	1.000000	0.972345	1.000000
16	1.000000	0.998747	0.994594	0.341716	0.999402
17	0.999999	0.999902	0.975562	0.222416	0.995140
18	0.999738	0.323224	1.000000	0.999087	1.000000

	16	17	18
16	1.000000		
17	1.000000	1.000000	
18	0.978131	0.931426	1.000000

Appendix 36

Comparison of body weights between ad lib liver-fed female and male adults of *Lucilia cuprina* using t-Test

	Female	Male
Mean	46.85	27.26
Standard Error	0.61	0.28
Median	49.11	26.76
Mode	49.09	28.05
Standard Deviation	7.43	3.41
Variance	55.22	11.66
Kurtosis	-0.44	-0.37
Skewness	-0.75	0.5
Range	32.1	15.04
Minimum	29.59	20.44
Maximum	61.69	35.48
Sum	7026.87	4089.55
Count	150	150

t-Test: Paired Two-Sample for Means

	Female	Male
Mean	46.85	27.26
Variance	55.22	11.66
Observations	150	150
Pearson Correlation	0.09	
Pooled Variance	2.22	
Hypothesized Mean Difference	0	
df	149	
t	30.35	
P(T<=t) one-tail	0.000000	
t Critical one-tail	1.66	
P(T<=t) two-tail	0.000000	
t Critical two-tail	1.98	

Appendix 37

Multiple comparisons of the mean values of ecdysteroid titres in the *ad lib* protein-fed female adults of *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

LEVELS ENCOUNTERED DURING PROCESSING ARE:
AGE

0	4	8	12	16	20	24	28	32	36	40
44	48	h								

DEP VAR: TITRE N: 65 MULTIPLE R: 0.734 SQUARED MULTIPLE R: 0.539

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	318.201808	12	26.516817	5.059592	0.000
ERROR	272.526798	52	5.240900		

COL/ ROW	AGE
1	0
2	4
3	8
4	12
5	16
6	20
7	24
8	28
9	32
10	36
11	40
12	44
13	48

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRE

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	-4.724000	0.000000			
3	-6.234400	-1.510400	0.000000		
4	-5.228800	-0.504800	1.005600	0.000000	
5	-6.924800	-2.200800	-0.690400	-1.696000	0.000000
6	-6.316800	-1.592800	-0.082400	-1.088000	0.608000
7	-7.019000	-2.295000	-0.784600	-1.790200	-0.094200
8	-8.479600	-3.755600	-2.245200	-3.250800	-1.554800
9	-8.285000	-3.561000	-2.050600	-3.056200	-1.360200
10	-8.239800	-3.515800	-2.005400	-3.011000	-1.315000
11	-8.048800	-3.324800	-1.814400	-2.820000	-1.124000
12	-7.940400	-3.216400	-1.706000	-2.711600	-1.015600
13	-7.722600	-2.998600	-1.488200	-2.493800	-0.797800

	6	7	8	9	10
6	0.000000				
7	-0.702200	0.000000			
8	-2.162800	-1.460600	0.000000		
9	-1.968200	-1.266000	0.194600	0.000000	
10	-1.923000	-1.220800	0.239800	0.045200	0.000000
11	-1.732000	-1.029800	0.430800	0.236200	0.191000
12	-1.623600	-0.921400	0.539200	0.344600	0.299400
13	-1.405800	-0.703600	0.757000	0.562400	0.517200

	11	12	13
11	0.000000		
12	0.108400	0.000000	
13	0.326200	0.217800	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.094519	1.000000			
3	0.004588	0.997529	1.000000		
4	0.034463	1.000000	0.999960	1.000000	
5	0.001061	0.944818	0.999999	0.992909	1.000000
6	0.003946	0.995944	1.000000	0.999907	1.000000
7	0.000879	0.926647	0.999997	0.988745	1.000000
8	0.000146	0.339865	0.936692	0.564709	0.996755
9	0.000157	0.421661	0.966877	0.656089	0.999107
10	0.000161	0.441736	0.972013	0.676815	0.999359
11	0.000183	0.529779	0.987403	0.760034	0.999870
12	0.000201	0.580981	0.992539	0.802918	0.999955
13	0.000255	0.682446	0.997858	0.876377	0.999997

	6	7	8	9	10
6	1.000000				
7	0.999999	1.000000			
8	0.951175	0.999204	1.000000		
9	0.975768	0.999560	1.000000	1.000000	
10	0.979801	0.999695	1.000000	1.000000	1.000000
11	0.991498	0.999948	1.000000	1.000000	1.000000
12	0.995185	0.999984	1.000000	1.000000	1.000000
13	0.998752	0.999999	0.999998	1.000000	1.000000

	11	12	13
11	1.000000		
12	1.000000	1.000000	
13	1.000000	1.000000	1.000000

LEVELS ENCOUNTERED DURING PROCESSING ARE:

AGE	48	52	56	60	64	68	72	76	80	84	88
		92	96	100	104	108	112	116	h		

DEP VAR: TITRE N: 90 MULTIPLE R: 0.727 SQUARED MULTIPLE R: 0.528

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	9271.965965	17	545.409763	4.745885	0.000
ERROR	8274.431671	72	114.922662		

COL/ ROW	AGE
1	48
2	52
3	56
4	60
5	64
6	68
7	72
8	76
9	80
10	84
11	88
12	92
13	96
14	100
15	104
16	108
17	112
18	116

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRE

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	-0.647200	0.000000			
3	5.348000	5.995200	0.000000		
4	8.749600	9.396800	3.401600	0.000000	
5	28.644200	29.291400	23.296200	19.894600	0.000000
6	37.799200	38.446400	32.451200	29.049600	9.155000
7	16.750800	17.398000	11.402800	8.001200	-11.893400
8	10.233800	10.881000	4.885800	1.484200	-18.410400
9	1.836600	2.483800	-3.511400	-6.913000	-26.807600
10	2.843800	3.491000	-2.504200	-5.905800	-25.800400
11	2.845600	3.492800	-2.502400	-5.904000	-25.798600
12	5.013600	5.660800	-0.334400	-3.736000	-23.630600
13	1.177800	1.825000	-4.170200	-7.571800	-27.466400
14	1.101400	1.748600	-4.246600	-7.648200	-27.542800
15	3.097000	3.744200	-2.251000	-5.652600	-25.547200
16	2.937200	3.584400	-2.410800	-5.812400	-25.707000
17	2.786200	3.433400	-2.561800	-5.963400	-25.858000
18	0.929800	1.577000	-4.418200	-7.819800	-27.714400

	6	7	8	9	10
6	0.000000				
7	-21.048400	0.000000			
8	-27.565400	-6.517000	0.000000		
9	-35.962600	-14.914200	-8.397200	0.000000	
10	-34.955400	-13.907000	-7.390000	1.007200	0.000000
11	-34.953600	-13.905200	-7.388200	1.009000	0.001800
12	-32.785600	-11.737200	-5.220200	3.177000	2.169800
13	-36.621400	-15.573000	-9.056000	-0.658800	-1.666000
14	-36.697800	-15.649400	-9.132400	-0.735200	-1.742400
15	-34.702200	-13.653800	-7.136800	1.260400	0.253200
16	-34.862000	-13.813600	-7.296600	1.100600	0.093400
17	-35.013000	-13.964600	-7.447600	0.949600	-0.057600
18	-36.869400	-15.821000	-9.304000	-0.906800	-1.914000

	11	12	13	14	15
11	0.000000				
12	2.168000	0.000000			
13	-1.667800	-3.835800	0.000000		
14	-1.744200	-3.912200	-0.076400	0.000000	
15	0.251400	-1.916600	1.919200	1.995600	0.000000
16	0.091600	-2.076400	1.759400	1.835800	-0.159800
17	-0.059400	-2.227400	1.608400	1.684800	-0.310800
18	-1.915800	-4.083800	-0.248000	-0.171600	-2.167200

	16	17	18
16	0.000000		
17	-0.151000	0.000000	
18	-2.007400	-1.856400	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	1.000000	1.000000			
3	0.999997	0.999984	1.000000		
4	0.997715	0.994822	1.000000	1.000000	
5	0.007960	0.005838	0.081897	0.260681	1.000000
6	0.000223	0.000208	0.001257	0.006557	0.996135
7	0.559336	0.491124	0.964064	0.999234	0.948200
8	0.987209	0.976647	0.999999	1.000000	0.389291
9	1.000000	1.000000	1.000000	0.999884	0.018753
10	1.000000	1.000000	1.000000	0.999987	0.029302
11	1.000000	1.000000	1.000000	0.999987	0.029325
12	0.999999	0.999993	1.000000	1.000000	0.071959
13	1.000000	1.000000	1.000000	0.999617	0.013879
14	1.000000	1.000000	1.000000	0.999565	0.013399
15	1.000000	1.000000	1.000000	0.999993	0.032691
16	1.000000	1.000000	1.000000	0.999990	0.030513
17	1.000000	1.000000	1.000000	0.999985	0.028577
18	1.000000	1.000000	1.000000	0.999424	0.012377

	6	7	8	9	10
6	1.000000				
7	0.182288	1.000000			
8	0.013260	0.999948	1.000000		
9	0.000326	0.747115	0.998602	1.000000	
10	0.000442	0.833736	0.999720	1.000000	1.000000
11	0.000442	0.833875	0.999721	1.000000	1.000000
12	0.001076	0.953708	0.999998	1.000000	1.000000
13	0.000272	0.682609	0.996587	1.000000	1.000000
14	0.000268	0.674842	0.996241	1.000000	1.000000
15	0.000482	0.852652	0.999823	1.000000	1.000000
16	0.000456	0.840859	0.999763	1.000000	1.000000
17	0.000433	0.829260	0.999691	1.000000	1.000000
18	0.000259	0.657228	0.995359	1.000000	1.000000

	11	12	13	14	15
11	1.000000				
12	1.000000	1.000000			
13	1.000000	1.000000	1.000000		
14	1.000000	1.000000	1.000000	1.000000	
15	1.000000	1.000000	1.000000	1.000000	1.000000
16	1.000000	1.000000	1.000000	1.000000	1.000000
17	1.000000	1.000000	1.000000	1.000000	1.000000
18	1.000000	1.000000	1.000000	1.000000	1.000000

	16	17	18
16	1.000000		
17	1.000000	1.000000	
18	1.000000	1.000000	1.000000

Appendix 38

Multiple comparisons of the mean values of ecdysteroid titres in the *ad lib* protein-fed female reproductive system (ovaries, oviduct, and associated organs) of *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

LEVELS ENCOUNTERED DURING PROCESSING ARE:
AGE

48	52	56	60	64	68	72	76	80	84	88
92	96	h								

DEP VAR: TITRE N: 65 MULTIPLE R: 0.904 SQUARED MULTIPLE R: 0.818

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	.102645E+06	12	8553.774518	19.485256	0.000
ERROR	.228273E+05	52	438.987017		

COL/ ROW	AGE
1	48
2	52
3	56
4	60
5	64
6	68
7	72
8	76
9	80
10	84
11	88
12	92
13	96

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRE

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	13.980000	0.000000			
3	60.774000	46.794000	0.000000		
4	99.230000	85.250000	38.456000	0.000000	
5	109.988000	96.008000	49.214000	10.758000	0.000000
6	120.456000	106.476000	59.682000	21.226000	10.468000
7	48.476000	34.496000	-12.298000	-50.754000	-61.512000
8	40.486000	26.506000	-20.288000	-58.744000	-69.502000
9	12.158000	-1.822000	-48.616000	-87.072000	-97.830000
10	8.324000	-5.656000	-52.450000	-90.906000	-101.664000
11	17.170000	3.190000	-43.604000	-82.060000	-92.818000
12	14.628000	0.648000	-46.146000	-84.602000	-95.360000
13	27.914000	13.934000	-32.860000	-71.316000	-82.074000

	6	7	8	9	10
6	0.000000				
7	-71.980000	0.000000			
8	-79.970000	-7.990000	0.000000		
9	-108.298000	-36.318000	-28.328000	0.000000	
10	-112.132000	-40.152000	-32.162000	-3.834000	0.000000
11	-103.286000	-31.306000	-23.316000	5.012000	8.846000
12	-105.828000	-33.848000	-25.858000	2.470000	6.304000
13	-92.542000	-20.562000	-12.572000	15.756000	19.590000

	11	12	13
11	0.000000		
12	-2.542000	0.000000	
13	10.744000	13.286000	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.997253	1.000000			
3	0.001916	0.042709	1.000000		
4	0.000129	0.000130	0.190294	1.000000	
5	0.000129	0.000129	0.026012	0.999793	1.000000
6	0.000129	0.000129	0.002473	0.921393	0.999844
7	0.030330	0.334500	0.999207	0.018763	0.001617
8	0.137034	0.727861	0.941983	0.003081	0.000305
9	0.999291	1.000000	0.029464	0.000130	0.000129
10	0.999986	1.000000	0.012961	0.000129	0.000129
11	0.983432	1.000000	0.078972	0.000133	0.000129
12	0.995816	1.000000	0.048614	0.000131	0.000129
13	0.658950	0.997335	0.408683	0.000234	0.000133

	6	7	8	9	10
6	1.000000				
7	0.000216	1.000000			
8	0.000137	0.999991	1.000000		
9	0.000129	0.261562	0.637979	1.000000	
10	0.000129	0.144886	0.442476	1.000000	1.000000
11	0.000129	0.485080	0.859947	1.000000	0.999974
12	0.000129	0.362985	0.757826	1.000000	0.999999
13	0.000129	0.936407	0.999017	0.991931	0.954651

	11	12	13
11	1.000000		
12	1.000000	1.000000	
13	0.999796	0.998305	1.000000

Appendix 39

Multiple comparisons of the mean values of ecdysteroid titres in the *ad lib* protein-fed female body without reproductive system of *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

```
LEVELS ENCOUNTERED DURING PROCESSING ARE:
AGE    48      52      56      60      64      68      72      76      80      84      88
               92      96      h
```

DEP VAR: TITRE N: 65 MULTIPLE R: 0.655 SQUARED MULTIPLE R: 0.429

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	871.154098	12	72.596175	3.250629	0.002
ERROR	1161.313880	52	22.332959		

COL/ ROW	AGE
1	48
2	52
3	56
4	60
5	64
6	68
7	72
8	76
9	80
10	84
11	88
12	92
13	96

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRE

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	1.716000	0.000000			
3	6.828000	5.112000	0.000000		
4	2.668000	0.952000	-4.160000	0.000000	
5	11.148000	9.432000	4.320000	8.480000	0.000000
6	9.220000	7.504000	2.392000	6.552000	-1.928000
7	7.668000	5.952000	0.840000	5.000000	-3.480000
8	3.022000	1.306000	-3.806000	0.354000	-8.126000
9	2.058000	0.342000	-4.770000	-0.610000	-9.090000
10	0.990000	-0.726000	-5.838000	-1.678000	-10.158000
11	-0.362000	-2.078000	-7.190000	-3.030000	-11.510000
12	1.516000	-0.200000	-5.312000	-1.152000	-9.632000
13	0.170000	-1.546000	-6.658000	-2.498000	-10.978000

	6	7	8	9	10
6	0.000000				
7	-1.552000	0.000000			
8	-6.198000	-4.646000	0.000000		
9	-7.162000	-5.610000	-0.964000	0.000000	
10	-8.230000	-6.678000	-2.032000	-1.068000	0.000000
11	-9.582000	-8.030000	-3.384000	-2.420000	-1.352000
12	-7.704000	-6.152000	-1.506000	-0.542000	0.526000
13	-9.050000	-7.498000	-2.852000	-1.888000	-0.820000

	11	12	13
11	0.000000		
12	1.878000	0.000000	
13	0.532000	-1.346000	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.999995	1.000000			
3	0.537848	0.881437	1.000000		
4	0.999160	1.000000	0.970947	1.000000	
5	0.024884	0.108973	0.961492	0.217000	1.000000
6	0.128265	0.389432	0.999821	0.601024	0.999982
7	0.356346	0.733386	1.000000	0.896395	0.993271
8	0.998164	1.000000	0.985625	1.000000	0.272462
9	0.999963	1.000000	0.923264	1.000000	0.141337
10	1.000000	1.000000	0.756689	0.999996	0.060312
11	1.000000	0.999959	0.456504	0.998118	0.017673
12	0.999999	1.000000	0.851693	1.000000	0.093074
13	1.000000	0.999998	0.576760	0.999721	0.029126

	6	7	8	9	10
6	1.000000				
7	0.999998	1.000000			
8	0.680682	0.935637	1.000000		
9	0.462669	0.800629	1.000000	1.000000	
10	0.255321	0.572175	0.999968	1.000000	1.000000
11	0.096854	0.288934	0.994749	0.999798	1.000000
12	0.349285	0.690751	0.999999	1.000000	1.000000
13	0.145566	0.390670	0.998961	0.999986	1.000000

	11	12	13
11	1.000000		
12	0.999986	1.000000	
13	1.000000	1.000000	1.000000

Appendix 40

Multiple comparisons of the mean values of ecdysteroid titres in the non protein-fed female adults of *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

LEVELS ENCOUNTERED DURING PROCESSING ARE:

AGE 0 4 8 12 16 20 24 28 32 36 40 44 48 h

DEP VAR: TITRE N: 65 MULTIPLE R: 0.757 SQUARED MULTIPLE R: 0.572

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	176.223780	12	14.685315	5.801745	0.000
ERROR	131.621840	52	2.531189		

COL/ROW	AGE
1	0
2	4
3	8
4	12
5	16
6	20
7	24
8	28
9	32
10	36
11	40
12	44
13	48

USING LEAST SQUARES MEANS. POST HOC TEST OF TITRE

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	-3.909000	0.000000			
3	-5.289400	-1.380400	0.000000		
4	-4.668800	-0.759800	0.620600	0.000000	
5	-5.914200	-2.005200	-0.624800	-1.245400	0.000000
6	-5.775400	-1.866400	-0.486000	-1.106600	0.138800
7	-6.020600	-2.111600	-0.731200	-1.351800	-0.106400
8	-5.690000	-1.781000	-0.400600	-1.021200	0.224200
9	-5.253400	-1.344400	0.036000	-0.584600	0.660800
10	-6.448000	-2.539000	-1.158600	-1.779200	-0.533800
11	-6.085600	-2.176600	-0.796200	-1.416800	-0.171400
12	-5.385600	-1.476600	-0.096200	-0.716800	0.528600
13	-6.660600	-2.751600	-1.371200	-1.991800	-0.746400

	6	7	8	9	10
6	0.000000				
7	-0.245200	0.000000			
8	0.085400	0.330600	0.000000		
9	0.522000	0.767200	0.436600	0.000000	
10	-0.672600	-0.427400	-0.758000	-1.194600	0.000000
11	-0.310200	-0.065000	-0.395600	-0.832200	0.362400
12	0.389800	0.635000	0.304400	-0.132200	1.062400
13	-0.885200	-0.640000	-0.970600	-1.407200	-0.212600

	11	12	13
11	0.000000		
12	0.700000	0.000000	
13	-0.575000	-1.275000	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.016038	1.000000			
3	0.000297	0.973991	1.000000		
4	0.001626	0.999902	0.999989	1.000000	
5	0.000144	0.732515	0.999988	0.988647	1.000000
6	0.000156	0.812613	0.999999	0.995955	1.000000
7	0.000139	0.664264	0.999935	0.977895	1.000000
8	0.000166	0.855117	1.000000	0.998099	1.000000
9	0.000321	0.978828	1.000000	0.999994	0.999978
10	0.000131	0.381684	0.993903	0.855950	0.999998
11	0.000137	0.620785	0.999841	0.968293	1.000000
12	0.000247	0.956995	1.000000	0.999947	0.999998
13	0.000130	0.264578	0.975300	0.740751	0.999919

	6	7	8	9	10
6	1.000000				
7	1.000000	1.000000			
8	1.000000	1.000000	1.000000		
9	0.999998	0.999892	1.000000	1.000000	
10	0.999973	1.000000	0.999905	0.992036	1.000000
11	1.000000	1.000000	1.000000	0.999749	1.000000
12	1.000000	0.999986	1.000000	1.000000	0.997233
13	0.999533	0.999984	0.998837	0.969877	1.000000

	11	12	13
11	1.000000		
12	0.999959	1.000000	
13	0.999995	0.986196	1.000000

LEVELS ENCOUNTERED DURING PROCESSING ARE:

AGE	48	52	56	60	64	68	72	76	80	84	88
		92	96	100	104	108	112	116	h		

DEP VAR: TITRE N: 90 MULTIPLE R: 0.604 SQUARED MULTIPLE R: 0.365

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	76.635337	17	4.507961	2.435343	0.004
ERROR	133.276148	72	1.851058		

COL/ ROW	AGE
1	48
2	52
3	56
4	60
5	64
6	68
7	72
8	76
9	80
10	84
11	88
12	92
13	96
14	100
15	104
16	108
17	112
18	116

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRE

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	0.248600	0.000000			
3	1.048600	0.800000	0.000000		
4	0.312600	0.064000	-0.736000	0.000000	
5	1.062200	0.813600	0.013600	0.749600	0.000000
6	-0.423600	-0.672200	-1.472200	-0.736200	-1.485800
7	-0.414000	-0.662600	-1.462600	-0.726600	-1.476200
8	-0.111800	-0.360400	-1.160400	-0.424400	-1.174000
9	0.411200	0.162600	-0.637400	0.098600	-0.651000
10	1.760200	1.511600	0.711600	1.447600	0.698000
11	1.725600	1.477000	0.677000	1.413000	0.663400
12	2.290200	2.041600	1.241600	1.977600	1.228000
13	-1.219600	-1.468200	-2.268200	-1.532200	-2.281800
14	-0.571600	-0.820200	-1.620200	-0.884200	-1.633800
15	-0.514800	-0.763400	-1.563400	-0.827400	-1.577000
16	-0.699200	-0.947800	-1.747800	-1.011800	-1.761400
17	0.170800	-0.077800	-0.877800	-0.141800	-0.891400
18	0.230800	-0.017600	-0.817800	-0.081800	-0.831400

	6	7	8	9	10
6	0.000000				
7	0.009600	0.000000			
8	0.311800	0.302200	0.000000		
9	0.834800	0.825200	0.523000	0.000000	
10	2.183800	2.174200	1.872000	1.349000	0.000000
11	2.149200	2.139600	1.837400	1.314400	-0.034600
12	2.713800	2.704200	2.402000	1.879000	0.530000
13	-0.796000	-0.805600	-1.107800	-1.630800	-2.979800
14	-0.148000	-0.157600	-0.459800	-0.982800	-2.331800
15	-0.091200	-0.100800	-0.403000	-0.926000	-2.275000
16	-0.275600	-0.285200	-0.587400	-1.110400	-2.459400
17	0.594400	0.584800	0.282600	-0.240400	-1.589400
18	0.654400	0.644800	0.342600	-0.180400	-1.529400

	11	12	13	14	15
11	0.000000				
12	0.564600	0.000000			
13	-2.945200	-3.509800	0.000000		
14	-2.297200	-2.861800	0.648000	0.000000	
15	-2.240400	-2.805000	0.704800	0.056800	0.000000
16	-2.424800	-2.989400	0.520400	-0.127600	-0.184400
17	-1.554800	-2.119400	1.390400	0.742400	0.685600
18	-1.494800	-2.059400	1.450400	0.802400	0.745600

	16	17	18
16	0.000000		
17	0.870000	0.000000	
18	0.930000	0.060000	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	1.000000	1.000000			
3	0.998856	0.999967	1.000000		
4	1.000000	1.000000	0.999990	1.000000	
5	0.998656	0.999959	1.000000	0.999987	1.000000
6	1.000000	0.999997	0.958193	0.999990	0.954719
7	1.000000	0.999998	0.960522	0.999992	0.957192
8	1.000000	1.000000	0.996190	1.000000	0.995648
9	1.000000	1.000000	0.999999	1.000000	0.999998
10	0.836631	0.947570	0.999994	0.963968	0.999995
11	0.856745	0.956990	0.999997	0.971060	0.999998
12	0.425087	0.629582	0.991989	0.681667	0.992885
13	0.993390	0.959175	0.442457	0.941308	0.431686
14	1.000000	0.999954	0.908733	0.999871	0.902830
15	1.000000	0.999983	0.930855	0.999948	0.925927
16	0.999995	0.999680	0.844007	0.999268	0.835907
17	1.000000	1.000000	0.999883	1.000000	0.999857
18	1.000000	1.000000	0.999956	1.000000	0.999944

	6	7	8	9	10
6	1.000000				
7	1.000000	1.000000			
8	1.000000	1.000000	1.000000		
9	0.999941	0.999950	1.000000	1.000000	
10	0.511103	0.519059	0.762328	0.981349	1.000000
11	0.539865	0.547878	0.786734	0.985572	1.000000
12	0.163181	0.167360	0.341360	0.757251	1.000000
13	0.999969	0.999964	0.997778	0.904152	0.076328
14	1.000000	1.000000	1.000000	0.999491	0.392868
15	1.000000	1.000000	1.000000	0.999763	0.437062
16	1.000000	1.000000	1.000000	0.997716	0.302100
17	1.000000	1.000000	1.000000	1.000000	0.921236
18	0.999998	0.999999	1.000000	1.000000	0.942187

	11	12	13	14	15
11	1.000000				
12	1.000000	1.000000			
13	0.084756	0.012721	1.000000		
14	0.419500	0.108334	0.999998	1.000000	
15	0.464755	0.127289	0.999995	1.000000	1.000000
16	0.325443	0.074120	1.000000	1.000000	1.000000
17	0.933858	0.564770	0.975093	0.999989	0.999996
18	0.952308	0.614840	0.963342	0.999966	0.999988

	16	17	18
16	1.000000		
17	0.999896	1.000000	
18	0.999749	1.000000	1.000000

Appendix 41

Comparison of ecdysteroid titres between ad lib liver-fed and non liver-fed female adults of *Lucilia cuprina* using t-Test

	Liver-fed female	Non liver-fed female
Mean	7.62	3.27
Standard Error	0.93	0.15
Median	3.93	2.9
Mode	3.08	4.85
Standard Deviation	11.43	1.82
Variance	130.74	3.32
Kurtosis	14.13	21.14
Skewness	3.58	3.51
Range	73.78	15.78
Minimum	1.13	1
Maximum	74.92	16.79
Sum	1142.61	490.66
Count	150	150

t-Test: Paired Two-Sample for Means

	Female	Male
Mean	7.62	3.27
Variance	130.74	3.32
Observations	150	150
Pearson Correlation	0.04	
Pooled Variance	0.76	
Hypothesized Mean Difference	0	
df	149	
t	4.62	
P(T<=t) one-tail	0	
t Critical one-tail	1.66	
P(T<=t) two-tail	0	
t Critical two-tail	1.98	

Appendix 42

Multiple comparisons of the mean values of ecdysteroid titres in the male adults of *Lucilia cuprina* using one-way analysis of variance and the Tukey test of hypothesis.

LEVELS ENCOUNTERED DURING PROCESSING ARE:

AGE	0	4	8	12	16	20	24	28	32	36	40
		44	48	h						

DEP VAR: TITRE N: 65 MULTIPLE R: 0.734 SQUARED MULTIPLE R: 0.538

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	363.544483	12	30.295374	5.054288	0.000
ERROR	311.687680	52	5.993994		

COL/ ROW	AGE
1	0
2	4
3	8
4	12
5	16
6	20
7	24
8	28
9	32
10	36
11	40
12	44
13	48

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRE

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	-4.617400	0.000000			
3	-5.475200	-0.857800	0.000000		
4	-6.169600	-1.552200	-0.694400	0.000000	
5	-7.650800	-3.033400	-2.175600	-1.481200	0.000000
6	-7.924800	-3.307400	-2.449600	-1.755200	-0.274000
7	-8.033200	-3.415800	-2.558000	-1.863600	-0.382400
8	-8.515200	-3.897800	-3.040000	-2.345600	-0.864400
9	-6.513800	-1.896400	-1.038600	-0.344200	1.137000
10	-8.723800	-4.106400	-3.248600	-2.554200	-1.073000
11	-8.818200	-4.200800	-3.343000	-2.648600	-1.167400
12	-8.705600	-4.088200	-3.230400	-2.536000	-1.054800
13	-8.091400	-3.474000	-2.616200	-1.921800	-0.440600

	6	7	8	9	10
6	0.000000				
7	-0.108400	0.000000			
8	-0.590400	-0.482000	0.000000		
9	1.411000	1.519400	2.001400	0.000000	
10	-0.799000	-0.690600	-0.208600	-2.210000	0.000000
11	-0.893400	-0.785000	-0.303000	-2.304400	-0.094400
12	-0.780800	-0.672400	-0.190400	-2.191800	0.018200
13	-0.166600	-0.058200	0.423800	-1.577600	0.632400

	11	12	13
11	0.000000		
12	0.112600	0.000000	
13	0.726800	0.614200	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	0.160828	1.000000			
3	0.042173	0.999997	1.000000		
4	0.012001	0.998308	1.000000	1.000000	
5	0.000663	0.753219	0.968797	0.998936	1.000000
6	0.000418	0.639179	0.927584	0.994694	1.000000
7	0.000346	0.591564	0.904195	0.991043	1.000000
8	0.000197	0.385371	0.750637	0.946115	0.999996
9	0.006189	0.989608	0.999972	1.000000	0.999927
10	0.000169	0.307380	0.664621	0.905086	0.999961
11	0.000161	0.275399	0.623617	0.881371	0.999904
12	0.000171	0.313793	0.672414	0.909288	0.999967
13	0.000318	0.565825	0.889875	0.988376	1.000000

	6	7	8	9	10
6	1.000000				
7	1.000000	1.000000			
8	1.000000	1.000000	1.000000		
9	0.999337	0.998619	0.983758	1.000000	
10	0.999998	1.000000	1.000000	0.964914	1.000000
11	0.999995	0.999999	1.000000	0.952446	1.000000
12	0.999999	1.000000	1.000000	0.967011	1.000000
13	1.000000	1.000000	1.000000	0.998028	1.000000

	11	12	13
11	1.000000		
12	1.000000	1.000000	
13	0.999999	1.000000	1.000000

LEVELS ENCOUNTERED DURING PROCESSING ARE:

AGE	48	52	56	60	64	68	72	76	80	84	88
		92	96	100	104	108	112	116	h		

DEP VAR: TITRE N: 90 MULTIPLE R: 0.440 SQUARED MULTIPLE R: 0.194

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
AGE	27.710930	17	1.630055	1.016727	0.451
ERROR	115.433054	72	1.603237		

COL/ ROW	AGE
1	48
2	52
3	56
4	60
5	64
6	68
7	72
8	76
9	80
10	84
11	88
12	92
13	96
14	100
15	104
16	108
17	112
18	116

USING LEAST SQUARES MEANS.
POST HOC TEST OF TITRE

MATRIX OF PAIRWISE MEAN DIFFERENCES:

	1	2	3	4	5
1	0.000000				
2	-0.354400	0.000000			
3	0.784200	1.138600	0.000000		
4	0.493400	0.847800	-0.290800	0.000000	
5	1.259200	1.613600	0.475000	0.765800	0.000000
6	0.571200	0.925600	-0.213000	0.077800	-0.688000
7	1.582400	1.936800	0.798200	1.089000	0.323200
8	1.261000	1.615400	0.476800	0.767600	0.001800
9	1.899400	2.253800	1.115200	1.406000	0.640200
10	0.369600	0.724000	-0.414600	-0.123800	-0.889600
11	1.125600	1.480000	0.341400	0.632200	-0.133600
12	0.736000	1.090400	-0.048200	0.242600	-0.523200
13	1.201200	1.555600	0.417000	0.707800	-0.058000
14	0.946400	1.302800	0.164200	0.455000	-0.310800
15	1.288200	1.642600	0.504000	0.794800	0.029000
16	0.804000	1.158400	0.019800	0.310600	-0.455200
17	1.573200	1.927600	0.789000	1.079800	0.314000
18	0.612200	0.966600	-0.172000	0.118800	-0.647000

	6	7	8	9	10
6	0.000000				
7	1.011200	0.000000			
8	0.689800	-0.321400	0.000000		
9	1.328200	0.317000	0.638400	0.000000	
10	-0.201600	-1.212800	-0.891400	-1.529800	0.000000
11	0.554400	-0.456800	-0.135400	-0.773800	0.756000
12	0.164800	-0.846400	-0.525000	-1.163400	0.366400
13	0.630000	-0.381200	-0.059800	-0.698200	0.831600
14	0.377200	-0.634000	-0.312600	-0.951000	0.578800
15	0.717000	-0.294200	0.027200	-0.611200	0.918600
16	0.232800	-0.778400	-0.457000	-1.095400	0.434400
17	1.002000	-0.009200	0.312200	-0.326200	1.203600
18	0.041000	-0.970200	-0.648800	-1.287200	0.242600

	11	12	13	14	15
11	0.000000				
12	-0.389600	0.000000			
13	0.075600	0.465200	0.000000		
14	-0.177200	0.212400	-0.252800	0.000000	
15	0.162600	0.552200	0.087000	0.339800	0.000000
16	-0.321600	0.068000	-0.397200	-0.144400	-0.484200
17	0.447600	0.837200	0.372000	0.624800	0.285000
18	-0.513400	-0.123800	-0.589000	-0.336200	-0.676000

	16	17	18
16	0.000000		
17	0.769200	0.000000	
18	-0.191800	-0.961000	0.000000

TUKEY HSD MULTIPLE COMPARISONS.
MATRIX OF PAIRWISE COMPARISON PROBABILITIES:

	1	2	3	4	5
1	1.000000				
2	1.000000	1.000000			
3	0.999933	0.993163	1.000000		
4	1.000000	0.999809	1.000000	1.000000	
5	0.980803	0.852092	1.000000	0.999952	1.000000
6	0.999999	0.999409	1.000000	1.000000	0.999989
7	0.870521	0.596766	0.999915	0.995810	1.000000
8	0.980535	0.650987	1.000000	0.999950	1.000000
9	0.630138	0.327559	0.994544	0.947814	0.999996
10	1.000000	0.999978	1.000000	1.000000	0.999643
11	0.993963	0.920896	1.000000	0.999997	1.000000
12	0.999972	0.995750	1.000000	1.000000	1.000000
13	0.987996	0.885206	1.000000	0.999984	1.000000
14	0.999200	0.973461	1.000000	1.000000	1.000000
15	0.976124	0.833730	1.000000	0.999919	1.000000
16	0.999906	0.991774	1.000000	1.000000	1.000000
17	0.875680	0.605004	0.999927	0.996196	1.000000
18	0.999998	0.998990	1.000000	1.000000	0.999996

	6	7	8	9	10
6	1.000000				
7	0.998239	1.000000			
8	0.999989	1.000000	1.000000		
9	0.968296	1.000000	0.999996	1.000000	
10	1.000000	0.986768	0.999633	0.898339	1.000000
11	1.000000	1.000000	1.000000	0.999944	0.999959
12	1.000000	0.999813	1.000000	0.991392	1.000000
13	0.999997	1.000000	1.000000	0.999987	0.999852
14	1.000000	0.999997	1.000000	0.999172	0.999999
15	0.999981	1.000000	1.000000	0.999998	0.999463
16	1.000000	0.999939	1.000000	0.995522	1.000000
17	0.998420	1.000000	1.000000	1.000000	0.987750
18	1.000000	0.998943	0.999995	0.976299	1.000000

	11	12	13	14	15
11	1.000000				
12	1.000000	1.000000			
13	1.000000	1.000000	1.000000		
14	1.000000	1.000000	1.000000	1.000000	
15	1.000000	1.000000	1.000000	1.000000	1.000000
16	1.000000	1.000000	1.000000	1.000000	1.000000
17	1.000000	0.999838	1.000000	0.999997	1.000000
18	1.000000	1.000000	0.999999	1.000000	0.999992

	16	17	18
16	1.000000		
17	0.999949	1.000000	
18	1.000000	0.999059	1.000000

Appendix 43

Comparison of ecdysteroid titres between ad lib liver-fed female and male adults of *Lucilia cuprina* using t-Test

	Female	Male
Mean	7.62	3.79
Standard Error	0.93	0.19
Median	3.93	3.39
Mode	3.08	2.09
Standard Deviation	11.43	2.36
Variance	130.74	5.56
Kurtosis	14.13	14.5
Skewness	3.58	3.11
Range	73.78	17.59
Minimum	1.13	1.1
Maximum	74.92	18.69
Sum	1142.61	568.42
Count	150	150

t-Test: Paired Two-Sample for Means

	Female	Male
Mean	7.62	3.79
Variance	130.74	5.56
Observations	150	150
Pearson Correlation	0.14	
Pooled Variance	3.85	
Hypothesized Mean Difference	0	
df	149	
t	4.13	
P(T<=t) one-tail	0.000030	
t Critical one-tail	1.66	
P(T<=t) two-tail	0	
t Critical two-tail	1.98	

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