Methods for the Assessment of the Effects of Chemicals on the Reproductive Function of Insects

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1 INTRODUCTION

Insect reproductive function is responsive to a complex of external factors—physical, chemical, biological. The effects of external stress are variable as the fertility of insects is usually very high and the range of potential aberrations very large. The anatomy of the reproductive organs is basically simple; therefore, morphological aberrations are distinguishable in quality as well as quantity. Insect ontogeny is also known in detail, thus dynamic observation of the response of affected reproductive organs is possible. The direct effects of external factors on individual developmental stages as well as delayed effects observable in embryogenesis of the succeeding generation can be experimentally examined. Embryonic development has been well described and changes are distinct. Many species produce several generations of offspring per year, so that the effects on several successive generations can be examined. In general, all actions of the adult organism are centred on reproduction. The condition of the reproductive organs and fertility of the individual thus indicate the physiological status of the whole organism as affected by external factors.

Insect reproduction has primarily been studied along the following lines:

1. population dynamics, especially the dynamics of pest outbreaks;
2. effects of sublethal doses of insecticides on fertility;
3. effects of chemosterilants and their application to insect control.

The effects of non-specific chemical pollutants in the natural environment on the reproduction of insects have not been sufficiently investigated, and reliable data are scarce. Considering that the study of insect reproduction and how it is regulated by chemicals can produce data of broad biological significance, it will be necessary to concentrate more effort on this line of research. Methods that have proved useful in the study of chemosterilants should also be employed.
2 CHANGES IN INSECT REPRODUCTION 
AND THEIR EXAMINATION

Chemicals can affect insect reproduction in any of its stages. Changes may appear in the following structures and/or functions:

1. female reproductive organs and oogenesis;
2. male reproductive organs and spermatogenesis;
3. number of laid eggs and hatchability;
4. embryogenesis;
5. mortality during postembryonic development and variations in mortality of the following generations; changes in development and mating habits;
6. mutagenic changes in chromosomes.

2.1 Female Reproductive Organs and Oogenesis

The condition of female reproductive organs is examined by dissecting fresh material by the usual methods. The abdomen is opened dorsally with fine scissors in physiological solutions (Pringle, Ringer or others). Fine organs are loosened by a stream of the solution from a hypodermic syringe. The usual fixatives (alcohol with acetic acid, Carnoy, Bouin, or others) may be used later, or some details can be emphasized by total staining (methylene blue, borax carmine). Whole mounts and smear preparations can be well observed using phase contrast or interference phase contrast. After fixation, histological preparations are made by the usual techniques, and sectioned materials stained with differentiating stains (recommended are Heidenhain's or Harris's haematoxylin, Pappenheim, etc.) or with specific histochemical stains (Feulgen for nucleic acids, Nile blue for lipids, mercury bromphenol blue for proteins).

Insect ovaries are paired organs situated in the abdomen consisting of ovarioles, ducts and accessory glands. Ovarioles are elongate tubes consisting distally of a germarium and proximally of a gradually formed vitellarium where oocytes grow and vitellogenesis takes place. Large numbers (up to several hundred) of ovarioles occur in Palaeoptera and Polyneoptera, several (mostly 4–7) in higher orders of insects. The germarium is the first to differentiate from primordial gonocytes (sometimes as early as during embryogenesis, e.g. in aphids). Later it contains mitotically dividing oogonia developing into oocytes or oocytes and trophocytes. The oocytes are enveloped in follicular epithelium and proceed towards the oviduct. Secretion of yolk and chorion, usually occurring prior to ovulation, is effected by follicular cells or trophocytes. According to the type of nutrition provided oocytes and corresponding structures, ovaries are categorized as atrophic (panoistic) without trophocytes in follicles, and meristic with trophocytes either in follicles (polytrophic ovaries) or in the germarium and connected with the oocyte by fine cytoplasmic cords (telotrophic ovaries).

Oviducts consist of lateral and common oviducts into which ovarioles open
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through the pedicels (interfollicular tissue), and the oviductus communis is usually differentiated into organs of copulatory and sperm-storing functions, the bursa copulatrix, spermatheca, etc. Accessory glands are either of mesodermal (mesadenia) or ectodermal origin (ectadenia). Their secretions, enveloping the egg, may participate in the formation of spermatophores, or lubricate the ovipositor or eggs.

Defects caused by chemicals appear in the germarium and vitellarium of individual follicles, in uneven development of follicles, and in accessory glands and outlets. The formation of spermatophores and motility of sperm may be affected. Compounds affecting development may impair the growth of oviducts and other organs. Sterilizing effects may be manifested by conspicuous changes in the germarium which may be smaller by one-half when compared with germarium of unexposed females due to inhibition of the division of oogonia and differentiation of oocytes and trophocytes (see reviews by Campion, 1972). Sections of panoistic ovaries show that the division of oogonia has been blocked, and already developed young oocytes have died and been resorbed. In polytrophic ovaries, oocytes as well as nutritive and follicular cells are affected. The nuclei of oocytes become pyonotic, oogonia cease to divide, and the development of egg chambers is blocked. In telotrophic ovaries, the zone of dividing trophocytes is the most damaged and, consequently, follicular cells do not divide (formation of compound egg chambers). Oocytes descend either irregularly or not at all. Changes in the vitellarium show in a reduced number of egg chambers, blocked growth of follicles or production of microfollicles. Vitellogenesis is often suppressed or entirely inhibited, so that the ovaries of older females cannot be distinguished from those of teneral ones (e.g. 6-azauridine in the housefly, Rezáková and Landa, 1967; Rezábová, 1968). Egg chambers may be resorbed.

All these changes result in a reduced number of laid eggs, and, if vitellogenesis is suppressed, in complete sterility of the female. Deposition of yolk granules, indicating that vitellogenesis has begun, is easily distinguished in squash preparations of the vitellarium; phase contrast or phase interference contrast is useful, as are some stains (e.g. fast green). Changes in follicles may not be macroscopically distinct, for only in cases of serious damage are there changes in the shape and colour of unfixed follicles. However, such changes are very distinct in histological preparations. Changes in shape are characterized by alteration of the originally round or oval follicle into an irregular form; changes in colour are conspicuous as darkening of the follicle and unfixed material often grows opaque (this should be distinguished from the opaqueness of postvitellogenic follicles due to yolk deposition).

It is very important to estimate the condition of the distal follicle (i.e. the oldest one, situated distal from the germarium and proximal to the oviduct). This follicle usually is the largest, and secretion of chorion should be macroscopically distinct at the time of oviposition; occasionally this secretion is also seen in other
follicles (especially in atrophic ovarioles). The absence of chorion or diminution of distal follicles indicate at least partial sterility. This does not exclude production of eggs by these follicles (e.g., microeggs in *Dixippus morosus* after treatment with juvenoids (Socha and Gelbíč, 1973).

Follicular epithelium and follicular cells are very susceptible to the action of chemicals. Uncoordinated division of nuclei may take place, intercellular membranes may disappear, and tumour-like tissue with irregular clusters of chromatin may develop. Nutritive cells of polytrophic ovaries are not usually affected in this way, but pycnotic changes occur in their nuclei.

Morphological and anatomical changes are also reflected in biochemical processes. The follicular content of nucleic acids increases due to changes in follicular epithelium. The effects of chemicals on the ovaries can also be examined by monitoring changes in the activity of mitochondria. Many compounds induce a manifold increase in mitochondrial activity.

Chemical effects on accessory glands become apparent in changes in their size and the altered colour of their secretions (in fresh material). In suitably fixed material (non-alcoholic fixatives, at best formalin), the amount of secretion can be macroscopically estimated and compared with that of control specimens. In cases where much secretion is deposited on the egg surface, it is possible to estimate gland function from this point of view. A lack of secretion or minor morphological changes in accessory gland might not affect fertility, but these factors can markedly affect the activation of sperm and thus, indirectly, fertility. Profound changes in accessory glands are always accompanied by changes in germinal tissues.

In species where spermatophores are formed either outside or in the female body, it is useful to estimate the number and condition of spermatophores in the bursa copulatrix of fertilized females. The lack of spermatophores is an indication either of male sterility or of changes in mating behaviour, but even the occurrence of more spermatophores than in the unexposed control does not ensure fertilization of the eggs in oviducts. The degree of differentiation of oviducts and outer genitalia should also be examined at dissection. Some biologically active compounds may upset differentiation of these organs and thus prevent the laying of otherwise normal eggs.

### 2.2 Male Reproductive Organs and Spermatogenesis

The preparation and observation of whole mounts and the techniques of histological processing have been described in the previous paragraph and are applicable to examination of male reproductive organs.

Insect testes are paired organs consisting of testicle tubules (testicular follicles), ducts and accessory glands. The ducts are connected with copulatory organs. The testicle tubules are elongate, vesicular or globular organs in which germinal cells divide, grow and differentiate. The tubules number several hundred in primitive
orders of insects (Palaeoptera, Polymetabola); in more advanced ones (Paraneoptera, Holometabola) there are usually only 4–7. Testicle tubules may be encased in a membranous sac (scrotum). They differentiate from primordial cells enveloped in connective and peritoneal tissues very early, often during embryogenesis or at the latest at the beginning of the pupal stage. Before the onset of spermatogenesis, the testicle tubules contain mitotically dividing spermatogonia. Some spermatogonia give rise to cyst cells with comparatively small nuclei and little cytoplasm. Spermatogonia are encased in cyst cells, divide in them and, together with the cells, produce cysts. Spermatocytes develop from spermatogonia. Spermatocytes of the first and second orders differ from spermatogonia in size, stainability of the nucleus and cytoplasm and their relative sizes. Reduction division of male gametes (meiosis) takes place in the spermatocyte stage. Spermatocytes develop into spermatids usually containing a large, easily stainable nucleus and almost no cytoplasm. These cells do not divide, but are gradually transformed into presperms and mature sperms. Cyst cells generally disintegrate only when the transformation has been completed and mature sperms descend into the seminal duct. Insect sperms, apart from a few exceptions, have one flagellum and a well-differentiated head (with a crystalline body in higher groups).

Three zones can be distinguished in the testicle tubule of a mature male: a distal zone of growth (encysted as well as free spermatogonia), a zone of reduction division (cysts with spermatocytes I and II), and, proximally, a transformation zone (cysts containing spermatids, presperms and sperms).

Ducts consist of two lateral seminal ducts and a common seminal duct passing into an ejaculatory duct of ectodermal origin and lined with a chitinous intima. Seminal vesicles serving as containers of sperm are situated at various places in the ducts. Accessory glands may be of mesodermal (mesadenia) or ectodermal origin (ectadenia). They consist of glandular and connective tissues and their secretions make up seminal fluid, sections for production of spermatophores, etc. Outer genitalia are mostly sclerotized and of very diverse shapes. Their structure, which may be relatively complex and of different nomenclature in different groups, primarily consists of an organically paired penis and variously modified gonapophyses.

Sterilizing effects on a testis are macroscopically distinct, especially as reduction in the size of testicle tubules as well as the whole testis and derangement of differentiation of the ducts and outer genitalia. However, sterilizing effects may be manifested in a reduced number of testicle tubules or deranged differentiation of testicle tubules from primordial cells, showing either in a marked reduction of the number of testicle tubules (down to 1/3 in Polyneoptera) or occurrence of irregularly shaped testicle tubules, which in fact represent several fused together. In contrast to the ovaries, a testis in which differentiation of the follicles has been disturbed need not mean complete sterility; such a state is even physiologically common in some groups (e.g., Collembolla). Considerable
differences in the size of the testis between normal and affected individuals are much more frequent; the whole testis may be reduced to one-third of its original length.

Toxic chemicals may impair differentiation of seminal ducts or development of the seminal ducts may be totally inhibited although germinal tissues have not been seriously damaged. The same consequence may occur with external genitalia. Passage of mature sperm through the ducts may be blocked, or mating prevented by defects in the copulatory organs. Macroscopic changes of accessory glands are manifested in the same fashion as in testicle tubules. Changes are primarily in size; however, the degree of filling of the ducts and seminal vesicles with mature sperm, and the amount and quality of secretion of the accessory glands must also be examined. Although the amount of sperm in the seminal ducts and seminal vesicles may not be a decisive indicator of fertility, pronounced differences in the size of seminal vesicles may be manifested in the mating ability of males, particularly in species where multiple mating is common.

Histopathological changes are often found with histological processing of the reproductive organs of males exposed to toxic chemicals. The germarium, apical part of the testicle tubule containing the zone of growth, may be shown to be damaged by the structure of the apical cell. Histopathological changes show mainly in disconnection or degeneration of cytoplasmic connections within spermatagonia which then become pycnotic, causing the whole apical cell to gradually disintegrate. The cell itself may vacuolate, or conspicuous resorptions may appear in its cytoplasm. Some compounds directly affect spermatogonia. Their effects show in inhibition of the mitotic division of spermatogonia, in disorders of the differentiation of cyst cells, or in incomplete encystation of secondary spermatogonia. Signs of necrosis, such as pycnosis of nuclei, disintegration of cytoplasm, formation of chaotic synctia, etc., may be found in spermatogonia of the apical zone.

A marked derangement of the proportioning of spermatocytes occurs in the zone of meiotic division (layer containing spermatocytes) when reduction division has been inhibited. Meiosis is blocked by all mitotic poisons. If there is no layer containing secondary spermatocytes in a testis where the transformation zone has already been differentiated, it always means that meiosis has been blocked. Changes in the number of encysted spermatocytes are frequent. In the cyst of control individuals, there should be an accurately delimited number of cells (2, 3, 8, 16, etc.). All cysts should contain approximately the same number of cells. Greater differences in the number of encysted spermatocytes are the result of inhibited differentiation and division of germinal cells. Complete sterility is manifested by disintegration and pycnosis of the nuclei of spermatocytes, loss of their originally globular shape, or premature disintegration of cysts.

In the transformation zone where cysts disintegrate and mature sperms develop, we should especially look for disintegrating cysts and morphological changes in sperm and presperm. Disorders in transformation may become
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apparent in premature disintegration of cysts containing immature sperm. However, these changes need not indicate sterility, because presperm may sometimes mature only in the seminal duct. Morphological defects are rarely found in sperm and presperm; these occur only when germinal cells have been seriously damaged (pycnosis, vacuolation). Malformations can be best estimated in mature sperm, e.g., the shape of head, which is elongate in most species, may be changed, or there are changes in differentiation of the flagellum. Malformed sperms are non-functional. Their percentage can be ascertained in squash preparations, or in a Bürker chamber by the same methods used for vertebrate sperm. All sperm with the flagellum should be motile; physiological immobility of insect sperm is connected with the loss of the flagellum. Individuals whose sperm is not motile are often sterile.

Adverse effects related to histopathological changes in germinal cells can also be found in other tissues of the male reproductive system. In accessory glands, the structures of secretion epithelium are often affected (reduction of cytoplasm, pycnosis and disintegration of nuclei, vacuolation of cells) and disorders in secretion ensue. Differentiation of epithelium is inhibited, or there is a chaotic formation of syncytia in cases of serious damage. Similar changes may appear in the epithelium of the seminal duct, in peritoneal tissues and in connective tissues of the testes. Cases of proliferation resembling tumour growth, starting in the connective tissue, have been shown.

Also, the time sequence in differentiation processes in germinal cells is most important to the study of changes in the reproductive system. Transformation may be shifted without noticeable histopathological defects, but males with immature sperm will be unable to fertilize females. Therefore, it is always necessary to define accurately that stage of ontogeny in which individual zones of testicle tubules develop in order to trace a possible disharmony in the timing of individual phases of spermatogenesis.

2.3 The Number of Laid Eggs and Hatchability

Assessing the number of laid eggs and their hatchability is essentially the only quantitative method revealing changes in fecundity and fertility of insects, and this reflects the adverse effects of chemicals on various phases of development of their reproductive organs.

Insect eggs are laid either individually or in batches. Oviposition is either single (i.e. only one mating and fertilization are followed by a single oviposition at which all eggs are laid), or multiple, when a single fertilization is followed by the laying of several batches of eggs. Repeated mating is rather rare. Parthenogenesis (the laying of unfertilized diploid eggs) is relatively widespread in insects and is especially common in primitive groups and in social insects. Partial parthenogenesis also occurs quite frequently.

It is easy to investigate fecundity if the methods for the rearing of the
experimental species have been well worked out. Experimental females should always be fertilized only by normal control males. If this is not possible (affected field populations), then at least by males affected to different degrees. If the purpose of the experiment is to ascertain through fertility of females to what extent the reproductive ability of males has been indirectly altered, it is necessary to mate the treated males with healthy females. The females must naturally be observed throughout their adult life because intervals between individual batches of eggs may be longer or otherwise different from those observed for control populations. It is useful always to examine a statistically significant sample of treated and control females, i.e., at least 30 individuals in each experiment. Besides the number of laid eggs, variations in their number per batch should also be noted, as should variations in the number of batches or changes in habitat preference for egg deposition and other comparable factors. The number of eggs laid under natural conditions can be evaluated only in species laying batches rather than individual eggs, and only in populations which have been demonstrably affected as a whole.

Many compounds do not alter the number of laid eggs but markedly affect their hatchability. It is possible to detect, in this way, changes in the fertility of males because unfertilized eggs do not hatch. Examination of hatchability is also the only reliable quantitative method for determining reproductive interference in parthenogenetic species in which fertilization does not affect the number of laid eggs. If incidental spontaneous or geographical parthenogenesis has been shown in a species, reproduction from such parthenogenetic individuals may distort data on the fertility of males, indirectly obtained through data on the number of eggs laid by normal females mated with the treated males. In some cases (e.g., after administration of juvenoids to Dixippus morosus, Socha and Gelbic, 1973), a paradoxical increase in oviposition per day may occur owing to irregularities in the maturation of individual follicles, but hatchability is minimal.

The causes of reduced hatchability vary. It is closely related to morphological defects of eggs (defective chorion enabling desiccation, defective microphyile, etc.). Hatchability may be reduced by an increase in the mortality of gametes or zygotes, by disorders in embryogenesis, and frequently by the inability of first instar larvae to leave the egg or break through the chorion.

2.4 Embryogenesis

The study of embryonic development of insects is a very subtle method of estimating the effectiveness of individual chemical agents interfering with reproduction. Changes in embryogenesis may appear even in those cases where mortality has not increased nor have adverse morphological changes in the development of reproductive organs occurred. The course of embryonic development can be observed either directly on live material or on fixed
specimens. Examination of live material is facilitated by the transparency of the chorion (e.g., in water, lactic acid, oil, etc.), and by using phase contrast, infrared light, or other techniques to increase resolution.

Eggs are fixed by placing them into solutions at regular intervals in order to record all stages of embryogenesis. Carnoy can be used for whole staining and Huettner or Bouin for histological processing. The chorion must be removed either mechanically or chemically with sodium hypochlorite for whole mounts stained with borax carmine differentiated in lactic acid, toluidine blue or other stains. Before histological processing the chorion should be removed or at least punctured for better penetration of reagents and embedding medium. Sections 4–6 μm thick are stained with Heidenhain’s, Harris’s or Mayer’s haematoxylin, Pappenheim, toluidine blue, etc. The most suitable fixation and staining should be tried beforehand for each species studied.

There are two main types of insect embryogenesis. The mosaic developmental type (Diptera) is characterized by early determination of embryonic structures. Damage to such eggs is most often revealed in malformation of or deficiency in the damaged structure. However, later determination and a considerable ability to compensate for or repair damaged structures are typical of the regulation type of development (Heteroptera). The two developmental types are not sharply delimited, the difference is rather in the time when development can still be somewhat regulated, or before which determination has already taken place. There are many intermediate examples (Lepidoptera, Coleoptera) between the two extreme types. Embryonic development is much shorter in dipteran eggs with their early determination than in eggs of those species where determination begins later.

Disorders in embryonic development may result from damage to gametes during oogenesis and spermatogenesis, or from the action of chemicals in the developing embryo. The final effect depends on the characteristics of the chemical as well as on the type of embryogenesis in the given species. The range of effects on eggs of the regulation type is much wider than in mosaic eggs because of their greater ability to repair induced abnormalities.

Damage to genetic material, i.e. embryonic lethal mutations, is most often manifested during cleavage division as a delayed effect. Karyokinesis is initiated by chromosomal aberrations during early cleavage division and, consequently, chromosomal bridges are formed. The bridges can be identified by cytogenetical methods in sections or squashes (stained with lacto-aceto-orceine). Species with diffuse centromeres are very resistant to formation of chromosomal bridges. Chromosomal fragments remain in the nuclei of daughter cells and their rearrangement gives rise to reciprocal translocations. Reciprocal translocations as well as deletions of local character (typical of Lepidoptera) often appear only in the advanced stages of embryogenesis.

Compounds producing effects of other kinds (e.g. antibiotics, analogues of insect hormones, etc.), which do not so drastically derange chromosomal
material, are effective rather on the level of regulation of gene activity. Development is not inhibited during cleavage division; however, disorders in germ band formation, gastrulation, and the development of malformed miniature embryos have been caused by such compounds. Physiologically exacting processes, such as invagination of the germ band, blastokinesis and hatching, are frequently disrupted.

Other developmental stages are affected by compounds with specific toxic effects (mitotic poisons, respiratory poisons, compounds damaging the deposition of cuticle, etc.).

The results of contemporary research have shown that if hatchability is reduced by a test compound, the study of embryogenesis may yield valuable additional data of considerable practical impact, such as the effective concentrations, efficacy of various methods of chemical application, determination of the more susceptible phases of embryogenesis, and the effects on duration of the different types of embryonic development.

2.5 Mortality during Postembryonic Development and Variations
in Mortality in the Following Generations; Changes in
Development and Mating Habits

To quantify reproductive-related mortality, it is first necessary to distinguish the proportion of deaths due to natural causes (always higher under natural conditions than in the laboratory) and that proportion of unnatural deaths caused by factors other than those interfering with reproduction in the parental generation. This task is not always easy. Increased mortality of first instar larvae of the first filial generation is common in experiments with toxic chemicals. This effect is due to defects in embryogenesis and hatching. Evidence of abnormality may sometimes be detected in the later instars. Higher mortality in filial generations is often induced genetically through lethal mutations. There is little data supporting the idea presently, but in several cases dominant lethal mutations have been induced by chemicals (for examples see LaChance and Riemann, 1964; Valkovic and Grosch, 1968).

Sterility may be indirectly attributed to changes in the length of development, most often by slowing its rate (occurrence of supernumerary larval instars in the case of juvenoids) or to changes in mating behaviour. Mating may be shortened by various compounds thus reducing the probability that fertilization will occur. There are also a large group of compounds which impair orientation in insects seeking the opposite sex through pheromone attractants.

2.6 Mutagenic Changes in Chromosomes

Mutagenic changes induced by chemical compounds (Fahmy and Fahmy, 1954) may affect somatic cells (somatic mutations) as well as germinal cells. Only one
chromatid or the whole chromosome may be damaged. Susceptibility to the
mutagenic effects of chemicals depends on the number and shape of chromo-
somes, types of centromeres and other properties of this chromosome and
specific chemical. Individual developmental stages also differ in their suscepti-
bility to mutagenic effects. It has been found that the number of genetic defects
(chromosomal aberrations) increases in proportion to increased doses of
effective compounds.

3 THE TESTING OF EFFECTS OF CHEMICALS ON
REPRODUCTION
IN MUSCA DOMESTICAL L. (DIPTERA, MUSCIDAE)

*Musca domestica* is a suitable species for use in testing of chemical effects on the
reproduction of insects. A genetically well-defined WHO strain can be used for
experimentation (WHO Standard Insect Strain Reference Centre, Pavia, Italy).

Houseflies are reared in a medium consisting of groats, yeast and milk (1000 g,
3 g, 1200 ml). Pupation takes place in the upper layer of the medium; pupae are
then transferred into cages where adults are fed a mixture of powdered low-fat
milk and sugar (1:1) and water. Oviposition medium, the same as used in larval
rearing, is placed via small cups into the cages after 6 days. The temperature of
the rearing room should be 25° ± 2°C, RH 60–70%, photoperiod 18 L : 6 D.

Chemicals can be administered to various developmental stages. However,
ovarian development in adult flies is the most suitable stage for the study of
adverse effects on reproductive function. Controls reared under the same
conditions are necessarily evaluated at the same time, and all parameters
examined for experimental flies are compared to those of control specimens.

3.1 Tests on Larvae

3.1.1 *In the Medium*

A test chemical is mixed with the standard larval medium (mg of compound per g
of dry medium) and a certain number of larvae of a defined age are placed into the
mixture. Effects on newly laid eggs can also be assessed. The following
characteristics are examined: survival of larvae; pupation; adult emergence;
fecundity and fertility of adults; state of ovaries and testes in individual stages of
the development of reproductive organs (at certain intervals); and
embryogenesis.

3.1.2 *Topical Application*

A test compound dissolved in acetone (1:10; 1:100; 1:1000) is applied to the
surface of the larval body (1–2 μl according to the size of the larva). Except for
larvae ready to pupate, experimental animals are then placed in fresh medium. The same parameters as listed in the previous case are again examined.

3.2 Tests on Pupae

Newly formed or light brown puparia are treated with compounds dissolved in acetone at the same concentrations as in larval treatment. The following characteristics are examined: adult emergence; fecundity and fertility of adults; state of ovaries and testes in individual stages of the development of reproductive organs (at certain intervals); and embryogenesis.

For examination of adverse effects on an adult that has not yet emerged, part of the pupal case can be removed at the cephalic end and 1 µl of the compound dissolved in acetone administered.

3.3 Tests on Teneral Adults

3.3.1 Compounds Administered in Water

Tap water solutions at concentrations of 0.1, 0.01 and 0.001% are administered to teneral adults and replenished every 3 days. The following characteristics are examined: fecundity and fertility of adults; state of ovaries and testes in individual stages of the development of reproductive organs (at certain intervals); embryogenesis; and fecundity and fertility of F₁ generation, if necessary. If the effects of the compound are conspicuous, experiments are repeated for detailed determination of morphological and anatomical changes in the growth and development of the ovaries. A suitable method for accurately defining the time it takes a toxic chemical to act and the manner of the compound's action is by injection of 2–16 µg of the chemical in physiological solution into the upper left quadrant of the thorax followed by repeated detailed examination as described above.

3.3.2 Compounds Insoluble in Water

Compounds insoluble in water can be tested by topical application of acetone or other non-toxic solutions in concentrations of 1:10, 1:100 and 1:1000 once to both sexes (2 µl) 6–10 hours after emergence from the pupa. The same characteristics as in section 18.3.3.1 are then examined.

3.4 Evaluation of Changes

Changes caused by chemicals administered to any of the developmental stages are currently evaluated throughout experiments according to the parameters given above. Naturally, evaluation of developing ovaries is of prime importance,
Figure 1 Sketches of internal reproductive organs of insects with details of female ovaries; male (1) and female (2) internal reproductive organs, accessory glands dotted; panoistic (3) (atrophic); meroistic–poltyrophic (4) and meroistic–telotrophic (5) types of insect ovarioles. Abbreviated terminology: te, testis with peritoneal envelop; fo, testicular follicles; ve, vasa efferentia; vd, vas deferens; sv, seminal vesicle; ed, ejaculatory duct; ag, accessory glands; ov, ovaries; ol, ovarioles; tf, terminal filament; lo, lateral oviduct; co, common (median) oviduct; vu, vulva; bc, bursa copulatrix with gland; sp, spermatheca; ge, germarium; vi, vitellarium; oc, oocytes; tp, trophocytes; fe, follicular epithelium; ic, interfollicular cells; cc, cytoplasmatic cords.
either by examining whole mounts or by histological methods. The ovaries are dissected at regular intervals (3, 6, 9, 12, 15, 18 and 21 days from the start of the experiment), whole mounts of ovarioles are prepared, and individual components of the ovariole as well as of the egg chamber are examined using phase, anophtal or interference contrast, and compared with the development of untreated flies.

3.4.1 Normal Development

Embryonic development in the housefly takes 13 hours under standard conditions. Eggs are laid in an inhibited first maturation division. When maturation division is complete, the male and female pronuclei fuse and cleavage division of the zygote takes place. Cleavage nuclei produced later migrate to the egg surface, forming periblast and, when cell membranes have developed, blastoderm. A cap of pole cells is formed at the posterior pole. Some of the cleavage nuclei remain inside the yolk as vitellophages. A germ band differentiates on the ventral side of the blastoderm. Stomodaenum and proctodaeum develop as well as the cephalic furrow and neural ridges. Organogenesis is connected with segmentation, the germ band is shortened, dorsal closure occurs and the head develops. Larval structures are completed and cuticle is deposited. The larva hatches when embryogenesis is finished (see Figure 18.4). Larval development lasts 5 days, pupal stage 7 days. The paired ovaries of a teneral fly consist of a germarium and vitellarium. Egg chambers develop in the germarium from oocytes, nutritive and follicular cells, and then enter the vitellarium. The whole ovariole is enveloped in tunica propria and by its pedicel enters the lateral oviduct (Figure 18.1). Only the first egg chamber and germarium are present in the housefly ovariole immediately after emergence (differentiation takes place in the final pupal stage). Yolk is accumulated in the oocyte during the next few days, the egg chamber rapidly grows, and the second egg chamber descends from the germarium. A mature egg enveloped in chorion is ready in the ovariole when yolk deposition begins in the second egg chamber (Figure 18.2).

Each of two testes is pyriform and is formed by one testicle tubule. Accessory glands are absent. Their function is replaced by secretory activity of epithelial cells in the ductus ejaculatorius (Figure 18.1). Mitotic division of spermatogonia and meiotic divisions of spermatocytes has already taken place in the larval stages. Spermatids and the first mature spermatozoa can be found in the testes of the pupa. The process continues in the adult. In the apical part of the testis there is a zone of primary spermatogonia while secondary spermatogonia are situated basally. The secondary spermatogonia are organized into cysts where development continues. On all sides of the testes there is a downward succession from the area of spermatogonia of primary spermatocytes to secondary spermatocytes, spermatids, and sperm (Figure 18.3).
3.4.2 Changes Induced by Chemicals

Administration of chemicals (either topical or in food) may affect the following developmental processes:

(1) survival of larvae. Larval growth in the medium larval mortality.
(2) pupation. Number of larvae which have pupated, shape of pupae (round, elongate, irregularly pigmented), their size (comparison of the weight of experimental and normal pupae is recommended in special cases).

(3) adult emergence. Number of emerging adults, conspicuous changes in their external morphology (nonstretched wings, etc.). Not yet emerging pupae are examined for the lid broken by head. The pupae that have not emerged can
Figure 4 Normal and chemically affected development of the embryo. *Musca domestica*: (1) formation of blastoderm, normal development (after 120 minutes); (2) development inhibited in the stage of a few cleavage nuclei (after 120 minutes). *Pyrhocoris apterus*: (3) invagination of the germ band, normal development (46 hours); (4) blastokinesis, normal development (110 hours); (5) embryo after blastokinesis, normal development (120 hours); (6) development inhibited at cleavage division; (7) development inhibited in the germ band stage; (8) embryo with a low degree of organization, cuticular structures prevail; (9) development of miniature malformed embryo.
be dissected in order to find out whether the pupa is empty (death during metamorphosis).

(4) Fecundity and fertility of adults. The number and hatchability of laid eggs (100 eggs are placed on filter paper in a Petri dish, larvae are counted 24 hours later; this is repeated after 48 hours and final hatchability is expressed in percent). The number of segmented and black non-hatching eggs is recorded. The number of eggs per female can be determined in comparison with the control (individual couples are kept separately).

(5) Embryogenesis. Morphological and anatomical evaluation of the state of development at various intervals, using whole mounts or histological preparations. Inhibition of development in early cleavage division, disintegration of nucleic material and later of the entire content of the egg. Death in more advanced stages of embryogenesis is exceptional (e.g. of fully developed larvae). Embryonic death is indicated by different coloration and stainability.

(6) Fecundity and fertility of F1 generation. In the case of strong mutagens, the effects of the chemicals on F1 generation can be investigated by determining hatchability.

(7) State of ovaries and testes during development. Reproductive organs dissected in physiological solution are first examined using a binocular, then a phase microscope. Histological preparations are made by the usual methods (Figure 18.4).

Administration of low doses of chemicals results in limited hatchability of eggs (or its controlled inhibition) while the normal structure of eggs and normal course of vitellogenesis is preserved. Embryonic development may be inhibited in various phases, an extreme case being the inability of a mature larva to leave the egg.

Changes of another kind are caused by the medium range of concentrations. Typically these changes occur in individual parts of the egg chamber causing the entire structure to change during development, and no eggs are laid. The follicular epithelium, which has an important function in transport and synthesis in the growing egg chamber, is highly susceptible to chemicals. Normally the epithelium is formed by one layer of cells, which do not divide or participate in the development of chorion during the last phase of vitellogenesis. Tested compounds must be administered within 24 hours from adult emergence if effects on follicular cells are to be fully manifested. Later treatment interferes with vitellogenic processes, but the second egg chamber is entirely unaffected. The nucleus of a follicular cell begins to divide within 4–5 days after application of the compound. Mitosis does not occur. The cell grows without dividing, while the nucleic matter continues to divide. The process always begins in the oocyte region and spreads throughout the follicular epithelium. A thick belt of follicular cells with active nuclei is always formed around the egg chamber. Nutritive cells are not affected by this multiplication; however, their nuclei undergo pycnosis or chromatin forms small clusters. Cytoplasm of the nutritive cells vacuolates.
When the tumour-like proliferation of follicular epithelium and nutritive cells within the oocyte disintegrate and are resorbed, only a folded vitelline membrane with small remnants of follicular cells remain at the place of the egg chamber. Almost all egg chambers in the ovary are affected in this way, only a small percentage show signs of yolk deposition with subsequent resorption often accompanied by bizarre shaping of the growing egg chambers. Proliferation also appears in the second egg chamber, but never to such a degree as in the first because of the second chamber's low activity at the time of treatment. The highest doses of chemicals totally inhibit ovarian growth, possibly causing degeneration and resorption at the end of development. However, these effects are occurring at near lethal doses (Figure 2).

Changes in the development of male reproductive organs become apparent in an altered shape of external genitalia, deranged differentiation of reproductive ducts, inhibited development of accessory glands, and disorders in spermatogenesis. Chemicals induce pycnosis of nuclei in spermatogonia, necrosis in spermatocytes, or development of abnormal sperm. These may be malformed in various ways, or immotile (examination of whole mounts). Squash preparations display chromosomal changes, such as translocations or chromosomal bridges. Treatment of adults generally produces changes in the development of sperm or, rarely, tumours in the testes or seminal ducts.

4 THE TESTING OF EFFECTS OF CHEMICALS ON REPRODUCTION IN PYRRHOCORIS APTERUS (L.) (HETEROPTERA, PYRRHOCORIDAE)

Adult specimens of Pyrrhocoris apterus L. are reared in special cages with nylon netting on the bottom, fed on linden seed and kept at 25 °C and 18-hour photoperiod. Eggs fall from the cages into Petri dishes and are collected at 30-minute intervals. They are then placed on filter paper in a Petri dish and incubated in a thermostatically controlled atmosphere until they hatch.

4.1 Tests on Larvae

A 1 µl drop of acetone solution corresponding to 10 µg with a known concentration of an organic test substance is applied to the first abdominal segment. Development of the reproductive apparatus is examined in connection with moulting into further stages and the fecundity and fertility of adults. Compounds soluble in water are also administered as tap water solutions to experimental insects. The same criteria are examined.

4.2 Tests on Teneral Adults

Chemical compounds are administered to females in the same way as to larvae. The following parameters are then examined: oogenesis; number of eggs per
4.3 Tests on Eggs

Either pure compounds or their solutions in acetone or oil (one drop corresponding to 10 μg of pure compound or solution diluted 1:10, 1:100, 1:1000) are applied with a fine glass stick to the anterior or posterior poles of eggs. Other eggs are dipped in the solution or put in contact with paper saturated with an acetone test chemical solution. Only the solvent is applied to control eggs.

4.4 Evaluation of Changes

4.4.1 Normal Development

The ovarioles of *Pyrhocoris apterus* are of the telotrophic type. Future gametes divide and differentiate into oocytes and nutritive cells in the germarium. Yolk is deposited in the vitellaria where oocytes are surrounded with follicular cells. Oocytes in telotrophic ovarioles are nurtured by nutritive as well as follicular cells. The nutritive cells remain in the anterior part of the ovariole, sending out cord-like projections through which nutrition flows to the oocytes. The oocyte grows and undergoes maturation division.

The male reproductive apparatus of *Pyrhocoris apterus* (L.) consists of a pair of testes, each of which is formed by seven testicle tubules opening into the seminal vesicle. Each seminal duct is divided by a fine membrane into apical germarium and a part containing cysts. One, sometimes two, apical cells occur at the apex of each testicle tubule and are conspicuous by a large nucleus rich in chromatin, surrounded by a thin layer of cytoplasm. Cytoplasm is surrounded by a group of pyriform cells, rosette-forming spermatogonia, also with large nuclei and rich granulation. Cysts contain groups of germinal cells of the same developmental stage: spermatogonia, spermatocytes I and II, and spermatids. Spermatozoids are formed in the posterior part of the testicle tubule.

Cysts containing primary spermatocytes have very small, markedly stained nuclei. The lower situated secondary spermatocytes have larger nuclei in which 12 chromosomes can be distinguished. The nuclei of spermatids have large nucleoli. Spermiogenesis takes place in the posterior part of the seminal duct. The first stages of transformation occur in cysts which later burst open, releasing bundles of spermatozoids.

Male and female pronuclei fuse after oviposition and completion of meiotic division. The zygote undergoes several cleavage divisions producing cleavage
nuclei. Some of these remain inside the yolk as vitellophages, while the others migrate to the egg surface forming surface periblast and, after formation of cellular membranes separating nuclei, a one-layer blastoderm. The germ band develops from the blastoderm on the ventral side of the egg, and later is invaginated. Segmentation of the embryo is connected with the development of embryonic layers. Stomodaeum and proctodaeum are formed, oral appendages and extremities begin to develop. Blastokinesis (revolution of the embryo) serves to facilitate the utilization of yolk. Dorsal closure then takes place and embryogenesis is completed. The entire embryonic development takes 168–180 hours at 25°C.

4.4.2 Changes Induced by Chemicals

The effects of chemicals appear in the formation of egg chambers. Ovarian development is inhibited and the number of laid eggs is reduced, or eggs differ in size. Inhibition of mitosis in the apical trophocytes results in depletion of the nutritive tissue of older females, followed by disorders in previtellogenesis and activation of oocytes. The ooplasm of young oocytes usually vacuolates. During the division of the prefollicular syncytium, young follicular or interfollicular cells may be deranged. The follicular epithelial cells are unable to produce the nutrients essential for yolk synthesis because of their limited development. Egg chambers often contain two or more oocytes (compound egg chambers) as a result of prefollicular tissue deficiencies caused by inhibition of mitosis in the tissue. Multiplied germinal elements can also arise from the original cystoblast by its supernumerary division.

Chemicals can affect testes morphology as well as spermatogenesis and the vitality of ripe sperm (aspermia or inactivation of sperm from sterilization should be considered). All stages of spermatogenesis can be affected. Results show that spermatogonia undergoing division are the most affected, less affected are young spermatocytes, and least of all the stages of spermateliosis. Whole testes become atrophic along with destruction of germinal cells. The induction of dominant lethal mutations and the effect of compounds on spermatogenesis are often followed by a reduced vitality of sperm.

Compound and small-sized eggs do not develop. With chemicals affecting genetic material, the development of some eggs is inhibited at cleavage division. The others develop until the stage of blastoderm formation, or invagination of the germ band. Aberrations can be observed in the formation of the embryo (absence of certain structures, asymmetric arrangement, clusters of non-differentiated cells, etc.). Malformed miniature embryos frequently occur, as well as inhibition of embryo development at blastokinesis. Also, fully developed larvae die in the eggs. Embryos which ceased to develop in one of the mentioned stages sometimes survive in the egg for quite a long time. Embryonic death shows
in a changed colouring of the eggs. Miniature malformed embryos often are abnormally strongly pigmented (Figure 4).

5 THE TESTING OF EFFECTS OF CHEMICALS ON REPRODUCTION IN SPODOPTERA LITTORALIS (BOISD.) (LEPIDOPTERA, NOCTUIDAE)

Spodoptera littoralis, the Egyptian cotton leaf worm, is a suitable, non-diapau sing species for laboratory rearing. The artificial diet used for this species is composed of beans (200 g), brewer’s yeast (20 g), agar (20 g), ascorbic acid (4 g), vitamin mixture (6 g), preserving solution (22 ml), sodium benzoate (1.5 g) and distilled water (1200 ml). The rearing method has been described in detail by Sehnal et al. (1976).

Tested compounds can be applied to larvae, pupae and adults either topically or injected in food or by fumigation (see section on Musca domestica).

5.1 Evaluation of Changes

5.1.1 Normal Development

The female reproductive system consists of two ovaries, each with four polytrophic ovarioles. Previtellarium and vitellarium consist of a chain of developing oocytes. Each oocyte contains 3–5 nutritive cells. The ovarioles develop during the pupal stage.

Testes are clear yellow, located dorsally in the fifth abdominal segment, kidney-shaped. In the last larval instar they are set closely together, surrounded with common epithelial tissue, so that prepupae, pupae and adults seem to be unitesticular. Each testis is formed by four pear-shaped testicular follicles. An apical cell can be found at the end of the testicle tubule around which spermatogonia (globular cells with large nuclei) are concentrated. The zone of encysted spermatogonia is situated dorsally. The whole development of male germinal cells through primary and secondary spermatocytes and spermatids takes place in the cysts. The process of spermatogenesis can be divided into the period of growth, two successive divisions, and a transformation period (Figure 3).

The development of male germinal cells proceeds practically throughout the larval and pupal stages. Spermatogonia multiply in early larval instars. The first primary spermatocytes are found in fourth instar larvae. The first secondary spermatocytes appear in the testes of the penultimate instar. All developmental stages of spermatozoids can be found in the testes of the last larval instar. Transformation of haploid spermatids into spermatozoa occurs in prepupal stages and the process continues in the pupa. More than 95% of mature
spermatozoa can be found in adults. Accessory glands, vasa efferentia and others differentiate in the pupa.

5.1.2 Changes Induced by Chemicals

The following changes can appear in females: total degeneration of ovaries, inhibition of mitotic divisions in the germarium, disorders in vitellogenesis, degenerative changes in nutritive cells, derangement of nutritive function, proliferation of follicular epithelium and vacuolation of oocytes. In males, pyknotic nuclei occur in spermatogonia, mitotic and meiotic divisions are deranged and abnormal sperm develop. Proliferation of testicular epithelium or of epithelial cells of spermiducts can occur as well. Degeneration of accessory glands or disorders in their secretory activity take place in some cases. There are also induced changes in chromosomes (chromosomal bridges or translocations, Figure 3).

6 BIBLIOGRAPHY


