

THE EFFECTS OF INDUCED TRIPLOIDY ON THE REPRODUCTION
OF THE RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)
AND THE NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

A Thesis presented for the degree of Doctor of Philosophy
to the University of Stirling

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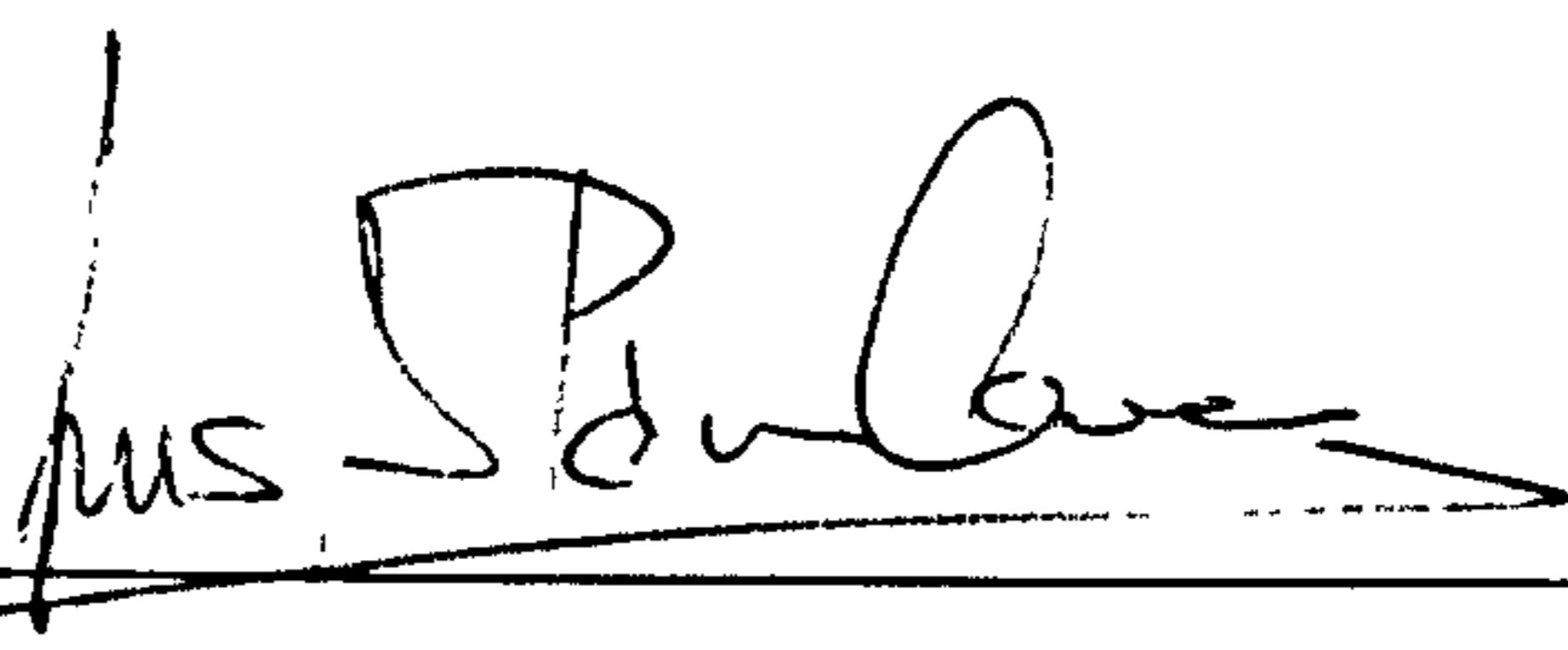
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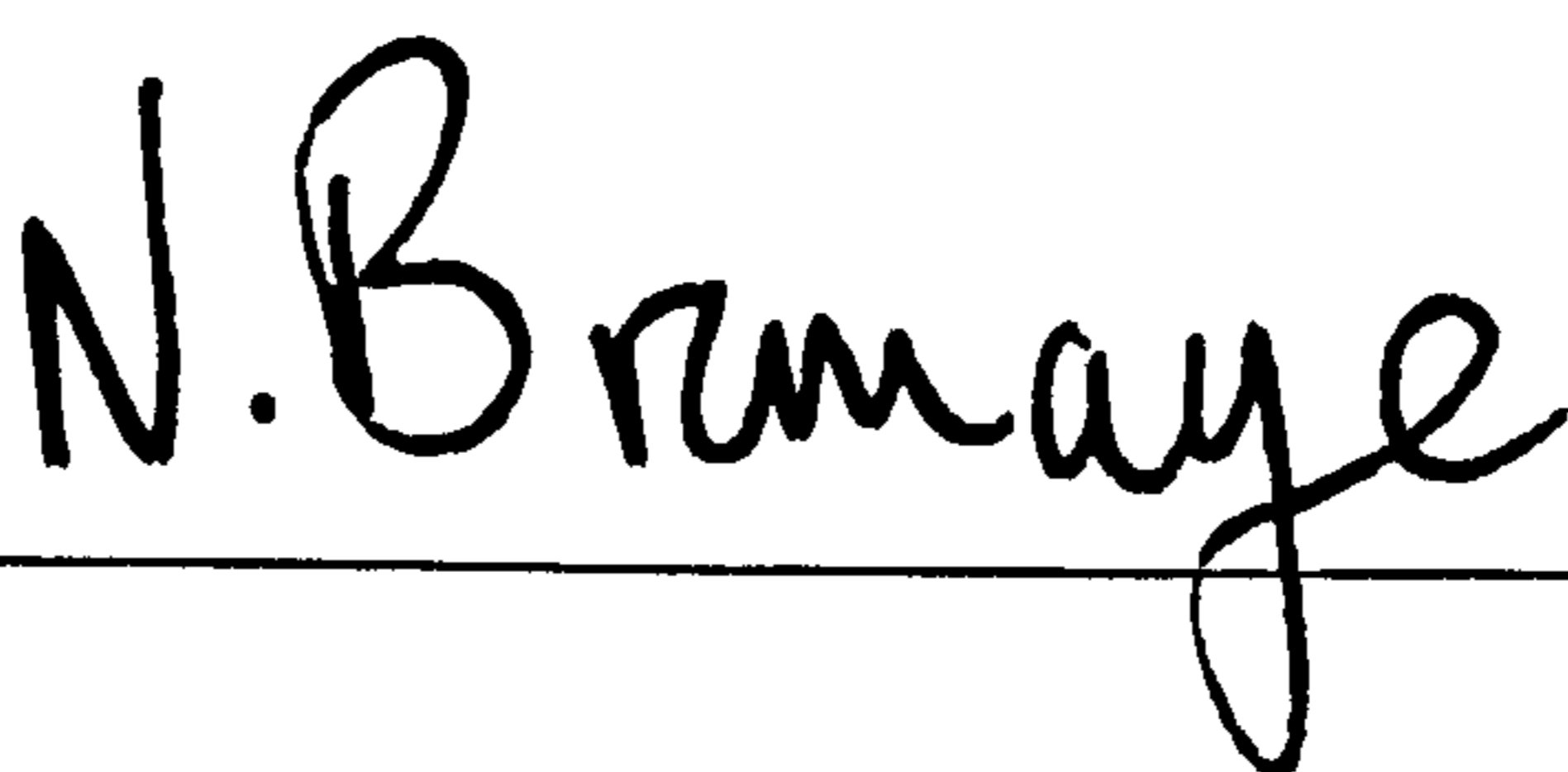
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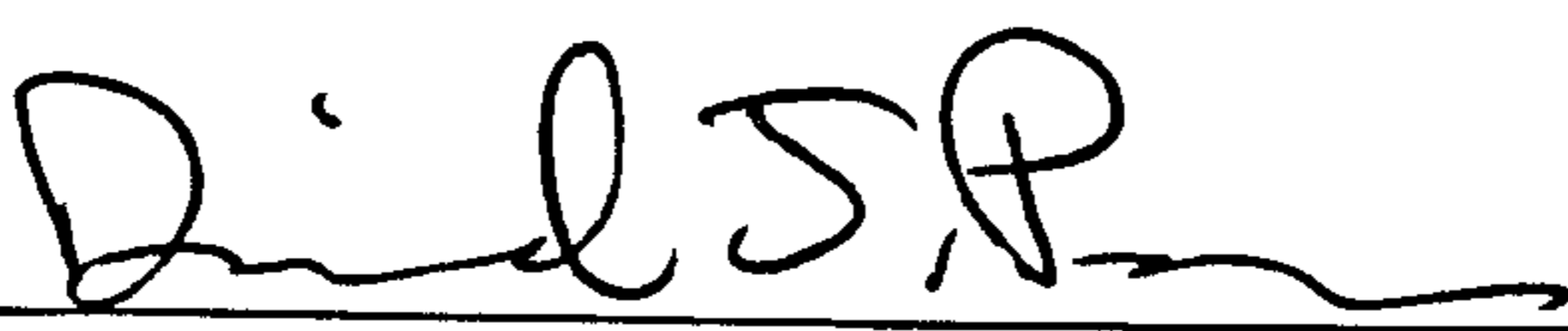
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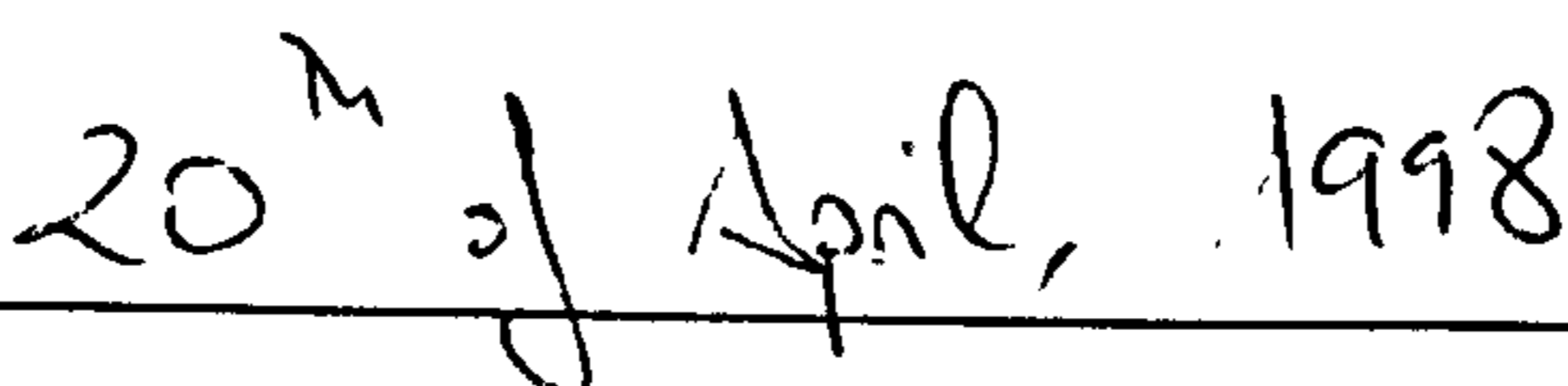
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PREFACE

This work is dedicated to my Parents, my Brother and Sister, who from the beginning (and especially in the final stages) encouraged me to complete it.

Esta obra está dedicada a mis Padres, mi Hermano y Hermana, que desde un principio (y especialmente al final) me animaron a completarla.

He revisado, al cabo de un año, estas páginas.

*Me consta que se ajustan a la verdad, pero en los primeros capítulos,
y aún en ciertos párrafos de los otros, creo percibir algo falso.*

*Ello es obra, tal vez, del abuso de rasgos circunstanciales,
procedimiento que aprendí en los poetas y que todo lo contamina de falsedad,
ya que estos rasgos pueden abundar en los hechos, pero no en su memoria...*

Creo, sin embargo, haber descubierto una razón más íntima.

La escribiré; no importa que me juzguen fantástico.

Jorge Luis Borges, El Inmortal.

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I will always be in debt to Professor Niall Bromage, who gave me the opportunity to write this Thesis.

ABSTRACT

Triploid rainbow trout produced by heat shock and control (diploid) siblings were raised separately at similar density, feeding and water quality regimes. No significant differences in body weight or condition factors were observed; however the weight of the eviscerated carcass was on average 20% higher ($P < 0.05$) in triploid fish compared to diploid fish at 20 and 44 months post-hatching.

The effects of triploidy on males were most evident during the final stages of spermatogenesis; in contrast, the gonadal development of triploid females was affected during its early stages, with the majority of the oogonia (30-70%) remaining within the oogonial clusters. A major finding was the presence of male-differentiating areas in most triploid females examined, which by the end of the sampling period appeared as gonadal hermaphrodites.

Testicular weight, gonado-somatic index, sperm cell density and spermatozoa motility were significantly lower in triploid than in diploid male siblings, although some triploid males produced viable progeny when crossed to normal (diploid) females. Characterisation of this progeny by image analysis of nuclear DNA revealed the presence of a near-triploid genome. A single 5 month-old juvenile had developed testes in meiotic phase, providing a first evidence for the generation of limited numbers of viable progeny by autotriploid rainbow trout males.

A cytogenetic analysis was carried out on monosex diploid and triploid populations of Nile tilapia. Synaptonemal complex analysis in diploid genotypes revealed the presence of an incompletely paired segment in the terminal region of the longest bivalent in heterogametic (XY) genotypes, which was not observed in homogametic genotypes. This unpaired region provides cytological evidence for the chromosomal basis of sex determination in *O. niloticus*. Meiotic analysis in triploids revealed the presence of longer ($P < 0.0001$) synaptonemal complexes in heterogametic (XXY) than in homogametic (XXX) genotypes, with a significantly different ($P < 0.0001$) nature of pairing evident between both groups. A model to explain the different progress in gametogenesis observed between male and female teleosts is discussed.

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LIST OF FISH SPECIES

<u>Common name</u>	<u>Latin name (Genus and Species)</u>
African catfish	<i>Clarias gariepinus</i>
Anchovy	<i>Engraulis mordax</i>
Arctic charr	<i>Salvelinus alpinus</i>
Asian seabass	<i>Lates calcarifer</i>
Atlantic halibut	<i>Hippoglossus hippoglossus</i>
Atlantic salmon	<i>Salmo salar</i>
Ayu	<i>Plecoglossus altivelis</i>
Barbel	<i>Barbus barbus</i>
Brook trout	<i>Salvelinus fontinalis.</i>
Brown trout	<i>Salmo trutta</i>
Carp	<i>Cyprinus carpio</i>
Channel catfish	<i>Ictalurus punctatus</i>
Cod	<i>Gadus morhua</i>
Coho salmon	<i>Oncorhynchus kisutch</i>
Eel	<i>Anguilla anguilla</i>
Fighting fish	<i>Betta splendens</i>
Flounder	<i>Platichthys flesus</i>
Gilthead seabream	<i>Sparus aurata</i>
Goldfish	<i>Carassius auratus</i>
Grey mullet	<i>Mugil cephalus</i>
Largemouth bass	<i>Micropterus salmoides</i>
Masu salmon	<i>Oncorhynchus masou</i>
Milkfish	<i>Chanos chanos</i>
Nile tilapia	<i>Oreochromis niloticus</i>
Pejerrey	<i>Odontesthes bonariensis</i>
Perch	<i>Perca fluviialis</i>
Pike	<i>Esox lucius</i>
Plaice	<i>Pleuronectes platessa</i>
Rainbow trout	<i>Oncorhynchus mykiss</i>
Red drum	<i>Sciaenops ocellatus</i>
Red sea bream	<i>Pagrus major</i>
Seabass	<i>Dicentrarchus labrax</i>
Smallmouth bass	<i>Micropterus dolomieu</i>
Sole	<i>Solea solea</i>
Striped bass	<i>Morone saxatilis</i>
Tench	<i>Tinca tinca</i>
Three-spined stickleback	<i>Gasterosteus aculeatus</i>
Turbot	<i>Scophthalmus maximus</i>

CHAPTER I

GENERAL INTRODUCTION

1.1 Introduction

Triploidy can be defined as a form of polyploidy characterised by the presence of three sets of homologous chromosomes in the genome, a condition naturally occurring in several species of plants, invertebrates, reptiles and amphibians (Fankhauser, 1945). Triploidy also occurs naturally in teleosts (Cherfas, 1966; Cimino, 1972; Cuéllar and Uyeno, 1972; Thorgaard and Gall, 1979). Lately, new lines of thought have emerged attributing to polyploidy the provision of additional, uncommitted loci necessary for major steps in the evolution of polyploid animals (Nagl, 1978; Brodskii, 1985).

Swarup (1956, 1959a, 1959b) first reported the artificial induction of triploidy and sexual development of triploid fish. He used the three-spined stickleback (*Gasterosteus aculeatus*) as an experimental model, treating the eggs with a heat shock shortly after fertilisation and rearing the embryos until sexual maturation. Several anomalies in their gonadal development were found, with the induction of triploidy differently affecting females and males. Females showed a reduction in ovarian size throughout life and microscopically, the ovaries from adult triploid fish contained numerous undifferentiated cells with the presence of only a few developing oocytes. The testes of juvenile triploids were similar to those of controls but, at maturity, appeared smaller and contained more connective tissue and cell-free spaces, the latter attributed to the degeneration of spermatocytes. No spermatozoa were observed (Swarup, 1959a). Since then several authors have described similar results in a wide variety of teleost species including bass and bream (Garret *et al.*, 1992; Sugama *et al.*, 1992), carps (Gervai *et al.*, 1980; Cassani and Caton, 1986), catfish (Wolters *et al.*, 1982; Manickam, 1991),

cichlids (Don and Avtalion, 1986; Penman *et al.*, 1987), flatfish (Purdom, 1972; Holmefjord, 1995), salmonids (Utter *et al.*, 1982; Benfey and Sutterlin, 1983; Chourrout, 1984), sturgeons (Vasetskii, 1967) and others (Kavumparath and Pandian, 1990).

Research on the induction and effects of triploidy in aquatic animals has been driven by the potential benefits offered by the manipulation of ploidy in these species. The primary goal pursued when generating triploid fish is their potential sterility, because of the multiple management options available with sterile populations. It has been generally accepted that the inability of triploid fish to undergo normal gametogenesis and produce viable gametes results from abnormal meiosis, in particular from difficulties in the process of chromosomal disjunction at the diakinesis stage of the first meiotic prophase. However, and in spite of the increasing application of the techniques for the induction of triploidy in commercially relevant aquatic species, no clear explanation of the differential effects of induced triploidy in male and female teleosts has been provided to date. Since the principles for the manipulation of ploidy in fish are based on the alteration of the meiotic process, a brief review of meiosis is included in this Chapter.

1.2 *An overview of meiosis*

The process of meiosis, unlike mitosis, reduces the amount of DNA. While mitosis produces daughter cells with the same genomic complement as the parental cell from which they originate, meiosis produces gametes with half the number of chromosomes found in somatic cells. During sexual reproduction, gametes combine at fertilisation to restore the somatic chromosomal complement. Unlike mitosis, in which each paternally and maternally derived chromosomes behave autonomously during cell division, meiosis is characterised by the pairing or synapsis of homologous chromosomes (John, 1990; Klug and Cummings, 1991; Lewin, 1997). The alteration of the normal meiotic events taking place immediately before or after fertilisation of female gametes is the basis for the techniques devised for the manipulation of ploidy in teleosts.

1.2.1 *First meiotic division*

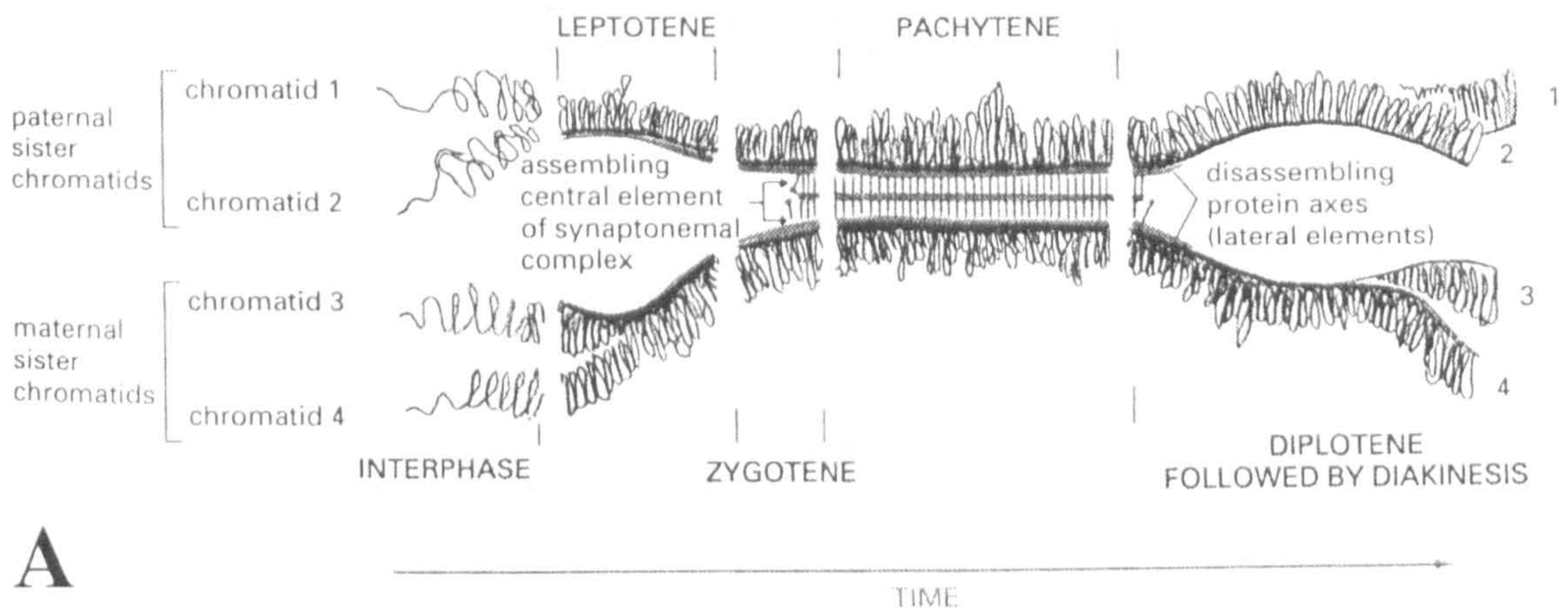
1.2.1.1 *Prophase*

As in mitosis, DNA replication precedes meiosis. Gonial cells entering the first meiotic division contain a $4c$ amount of DNA (c = the amount of DNA found in the haploid genome), and receive the name of *primary oocytes*. The first meiotic prophase is divided into five substages: *leptotene*, *zygotene*, *pachytene*, *diplotene* and *diakinesis*. During *leptotene* (from the Greek *lepto*, thin, and *tenia*, strings), the chromosomes of the primary oocytes begin to condense and become visible under the light microscope (John, 1990). In *zygotene* (*zygo*, to bring a pair together, to unite) homologous chromosomes come close to each other and begin to undergo zipper-like pairing or synapsis resulting in the formation of *bivalents*. Pairing is

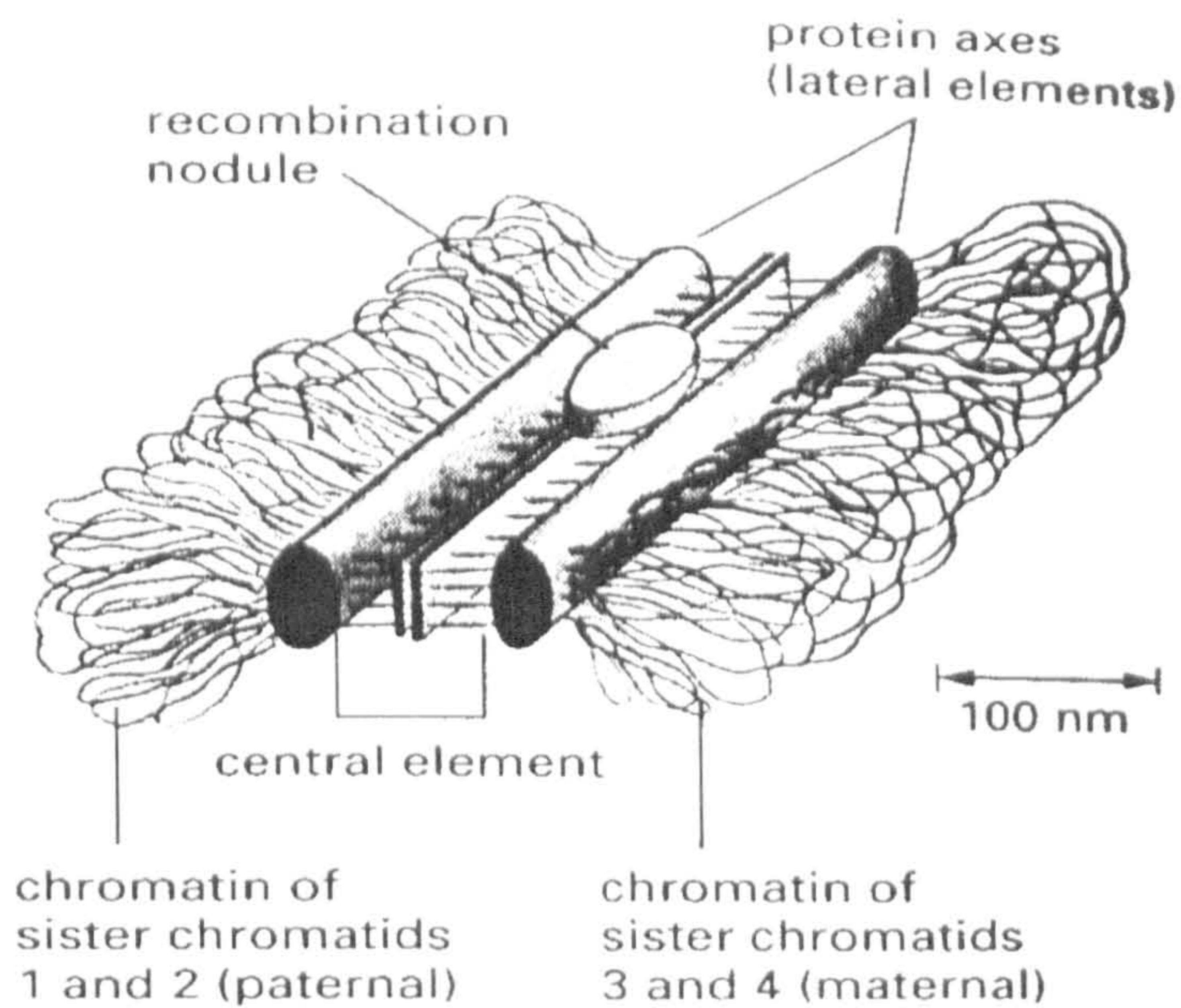
accompanied by the formation of a tripartite proteic scaffold between homologous chromosomes, the synaptonemal complex (Von Wettstein *et al.*, 1984; Figure 1.1, A). Chromosome synapsis proceeds by the establishment of intimate associations between homologous AEs (usually near the telomeres), which once assembled into the SC are then referred to as *lateral elements* (LEs). The area between two LEs is called the *central region*, where densely staining, spheroidal structures called *recombination nodules* can be observed (Von Wettstein *et al.*, 1984; Figure 1.1, B). These nodules are thought to be the cytological visualisation of large protein complexes involved in the enzymology of recombination, since their frequency and distribution correspond to the frequency and distribution of crossovers (Carpenter, 1988).

In pachytene (*pachy*, thick), coiling and packaging of the DNA and associated proteins forming the chromosomes continues. It is during this stage when it first becomes evident that each chromosome consists of a double structure, providing visual evidence for the replication of DNA (John, 1990). As in mitosis, the two DNA double helices are connected by a common centromere and receive the name of *sister chromatids*. Each bivalent contains two pairs of sister chromatids, and is sometimes referred to as a *tetrad* (Klug and Cummings, 1991).

While not visually evident during pachytene, *crossing over*, a major meiotic event, occurs during this stage, preceded by the synthesis of an amount of DNA constituting less than 1% of the total in the nucleus (Stern and Hotta, 1974; Hotta *et al.*, 1984). Crossing over, a critical source of genetic variability through which new combinations of genetic material arise, is the reciprocal exchange of DNA between non-sister chromatids, and takes place within the tetrad.



A



B

Figure 1.1

A, Time course of chromosome synapsis and desynapsis during meiotic prophase I.

B, Cross section of synaptonemal complex during the pachytene stage of prophase I showing the lateral and central elements with a recombination nodule (Alberts *et al.*, 1994).

The points of exchange become apparent as cross-like structures named *chiasmata* in the following stage, diplotene (*diplo*, double). Although the physical exchange between chromosome arms occurs during previous prophase substages, visible confirmation of crossing over is only possible when duplicated chromosomes begin to separate or desynapse (Loidl, 1994; Lewin, 1997).

During diakinesis (*dya*, separation, severance; *kinesis*, movement) the chromosomes further separate, and as this separation proceeds, the chiasmata are laterally displaced towards the end of the tetrads, a process known as *terminalisation* which begins in late diplotene and is completed during diakinesis (Murray and Szostak, 1985). It is also during diakinesis when the breakdown of the laminar components in contact with the inner membrane of the nuclear envelope commences, and the two centromeres in each tetrad become attached to the recently formed *spindle fibres* (Gerace and Burke, 1988). These fibres consist of microtubules, which are in turn formed by the assembly of multiple protein dimers consisting of two closely related polypeptides, α - and β -tubulin (Soifer, 1986; Sawin, 1993).

Microtubules are dynamic structures: the assembled tubule is in equilibrium with a cytosolic pool of tubulin subunits, and there is a continuous flux of tubulin in and out of the assembled form (Soifer, 1986; Mitchison, 1988; Hyams and Lloyd, 1994). Accessory proteins or MAPs (microtubule-associated proteins) influence the state of equilibrium. This dynamic equilibrium is an important aspect of microtubule function, allowing the tubules to disassemble and reassemble in response to cellular conditions (Sawin, 1993; Hyams and Lloyd,

1994). Two types of microtubules have been identified in the spindle fibres. Those called *kinetochore microtubules* have one end in one of the cytoplasmic poles, near the centrosomal (or centriolar region), and the other end anchored in the *kinetochore*, a region within the centromere of the chromosome formed by the interaction of special proteins with particular repetitive sequences of DNA (Murray and Szostak, 1985; Mitchison, 1988). The second type of microtubules are called *non-kinetochore microtubules*, which grow from the centrosome but have their other ends free. Non-kinetochore microtubules interdigitate with one another providing a framework to the spindle and maintaining the separation of the two cellular poles during chromosome disjunction (Murray and Szostak, 1985; Gerace and Burke, 1988; Mitchison, 1988; Sawin, 1993; Lewin, 1997).

1.2.1.2 *Metaphase, anaphase and telophase*

At the metaphase of the first meiotic division the chromosomes have maximally shortened and thickened, and the terminal chiasmata of each tetrad are visible as the only points of contact between non-sister chromatids (Klug and Cummings, 1991; Loidl, 1994). Each tetrad interacts with the spindle fibres and tubulin polymerisation resumes, facilitating their movement towards the equator of the cell and resulting in the formation of a unique configuration called *metaphase plate* (Sawin, 1993). During the first division a single centromere holds each pair of sister chromatids together. It does not divide (John, 1990). At the anaphase, one half of each tetrad (one pair of sister chromatids) is pulled towards each pole of the dividing cell. The products of the separation, or disjunction of the tetrads are two *dyads*, which are pulled by the kinetochore filaments to one or the other

cellular pole at random (Murray and Szostak, 1985; Hyams and Lloyd, 1994). This random segregation of the dyads is the basis for the Mendelian principle of independent assortment. The *telophase* of the first meiotic division is marked by the reconstitution of the nuclear membrane around the dyads. The two daughter cells resulting from the first meiotic division in females receive equal amounts (2c) of DNA and unequal amounts of cytoplasm. Almost the totality of the cytoplasm of the primary oocyte remains in one of the daughter cells, the *secondary oocyte*. The other daughter cell receives a minimum amount of cytoplasm and receives the name of *first polar body*. The accumulation of cytoplasm in one of the daughter cells is necessary for the nourishment of the developing embryo following fertilisation (Klug and Cummings, 1991; Lewin, 1997).

1.2.2 Second meiotic division

A second division of the sister chromatids is essential to achieve haploidy in the cellular products resulting from the meiotic process. In the majority of female teleosts, the nucleus of the secondary oocyte remains arrested at this stage until ovulation, and progress into the second meiotic division is only resumed upon fertilisation (Masui, 1985). During the second meiotic *prophase*, each dyad consists of one pair of sister chromatids attached by a common centromere. Breakdown of the laminar components forming the nuclear envelope of the secondary oocyte and the formation of a new spindle take place during this phase (Gerace and Burke, 1988). Polymerisation of tubulin subunits yields new microtubules that bind to the kinetochores in the dyads (Murray and Szostak, 1985; Soifer, 1986; Hyams and Lloyd, 1994).

During the second meiotic *metaphase*, the centromeres are directed to the metaphase plate by interaction with the kinetochore microtubules. In the second meiotic *anaphase*, kinetochore microtubules retract and pull the dyads in opposite directions, a process accompanied by the division of the centromere (Mitchison, 1988). The second meiotic *telophase* reveals one-half of each dyad, named *monads*, present at each pole (Murray and Szostak, 1985; John, 1990). During this final stage, the nuclear envelope is restituted around each daughter cell, and the cytoplasm of the secondary oocyte again divides unequally, producing an *ootid* (*ovum* in teleost females) and a *second polar body*, each of them containing a 1c amount of DNA in their nuclei (Gerace and Burke, 1988). The ovum initiates development as a zygote containing a 2c amount of DNA (1c from the ovum pronucleus and 1c from the spermatozoon nucleus)(Klug and Cummings, 1991).

1.3 Ploidy manipulation in teleosts

Two different cell divisions are disrupted for manipulating fish ploidy, depending on the time at which the treatment is applied after fertilisation. *Early shocks* inhibit the extrusion of the second polar body at the end of the second meiotic division. The outcome is a triploid zygote that contains the genome present in the ovum, the genome present in the second polar body, and the genome of the spermatozoon. *Late shocks* inhibit the first mitotic division (first cleavage) of the fertilised egg. The outcome is a zygote in which DNA has been replicated and contains a doubled number of chromosomes. Late shocks result in the generation of tetraploid individuals (Purdom, 1983; Thorgaard, 1983; Ihssen *et al.*, 1990). Strategies for sex control are frequently used in conjunction with ploidy manipulation techniques. Inactivation of the DNA present in the male gametes (spermatozoa) will result in the production of individuals whose genome is of exclusively maternal origin (gynogenotes). Similarly, inactivation of the DNA present in female gametes (ova) will result in the generation of individuals whose genome is of exclusively paternal origin (androgenotes). For the restitution of diploidy in the resulting zygote, DNA inactivation must be coupled with the inhibition of the extrusion of the second polar body or inhibition of the first mitotic division for successful gynogenesis, or suppression of the first mitotic division in androgenesis. The manipulation of meiosis or mitosis and the inactivation of the genome in fish may thus result in the production of triploid, tetraploid, gynogenetic or androgenetic individuals (Mair, 1993; Hussain, 1996).

1.4 *Fundamentals of ploidy manipulation*

A key point in the manipulation of plant or animal genomes is the clarification of the cellular mechanism(s) affected by the treatments used for ploidy manipulation. In general, techniques for ploidy manipulation are aimed to cause the non-disjunction of chromosome sets during cell division. It has been suggested that the treatments inhibit the polymerisation of tubulin dimers into microtubules, either by altering the kinetics of the equilibrium established between dimers and free microtubules or affecting the function of MAPs. These techniques may also affect the synthesis or function of other structural proteins (e.g. actin, kinesin, dynein), the assembly or function of ribosomal subunits and thus, protein synthesis, or the architecture and/or function of other cellular organelles (e.g. kinetochore, centrosomes, nuclear envelope and/or cellular membranes)(Soifer, 1986; Cleveland, 1988; Livezey, 1992; Sawin, 1993; Hyams and Lloyd, 1994; Rutberg, 1996).

Alternatively, the effects of the treatments applied for the manipulation of ploidy in teleosts may bear some relation to the dynamics of heat shock in vertebrate cells (Forristall, 1989; Hightower and Nover, 1991; Nover, 1991). In spite of the success of the techniques in use, the intimate molecular alterations involved in the manipulation of ploidy remain largely unclarified.

1.5 Techniques for the generation of triploid fish

Techniques for the generation of triploid fish are usually classified as *direct* or *indirect* methods. Direct methods are those in which the treatment is applied to the cells that will develop into triploid individuals. They include the use of chemicals, changes in temperature and pressure shocks. Indirect methods are those in which a polyploid progeny is generated first (by the use of direct methods) and is then used in different crosses devised to obtain the desired triploid individuals. Both direct and indirect methods are generally used in combination with sex-reversal strategies aimed to the production of monosex triploid progenies (Lincoln and Scott, 1983; Quillet *et al.*, 1991; Galbreath and Thorgaard, 1995; McCarthy *et al.*, 1996).

1.5.1 Direct methods

1.5.1.1 Chemical methods

A number of chemicals have been used to manipulate the ploidy of amphibians and fish. Several substances have been used in teleosts, such as colchicine (Smith and Lemoine, 1979), cytochalasin B (Refstie *et al.*, 1977), nitrous oxide (Sheldon *et al.*, 1986), and several anaesthetics (Johnstone *et al.*, 1989). Although they have proved very useful for research purposes, their tumorigenic and cytotoxic effects generally restrict their use.

1.5.1.2 Temperature treatments

Heat (Chourrout, 1980; Chourrout and Itskovitch, 1983; Johnstone, 1985; Don and Avtalion, 1986; Hussain *et al.*, 1991, Quillet *et al.*, 1991) or cold shocks (Valenti, 1975, Chourrout, 1980; Richter, 1987; Linhart *et al.*, 1991) have been

successfully applied in the generation of triploids of several teleost species. They can be defined as sudden changes of temperature generally within a range of 10 to 20 °C applied to the eggs shortly after fertilisation. The duration and time of application of these temperature shocks is species-specific, but generally varies between a 5-10 min (for heat shocks) and a 30-45 min (for cold shocks) treatment applied 5 to 90 min after fertilisation. Heat shocks are the most common treatments currently being used by the industry due to their simplicity, cost-effectiveness and efficiency, although egg quality appears to be a critical factor in the success of triploidy induction (Díaz *et al.*, 1993).

1.5.1.3 Pressure shocks

Compared to temperature shocks, pressure shocks offer the advantage of a more uniform treatment of the eggs, overcoming difficulties in heat transfer, particularly in species with large egg volumes. The duration of pressure shocks is also generally shorter compared to temperature treatments, thus minimising mortalities (Chourrout, 1984; Lou and Purdom, 1984; Lincoln, 1989; Hussain *et al.*, 1991). This might explain why triploid yields are usually higher with pressure than with temperature shocks, although abnormal embryo development and chromosomal aberrations have been described following the application of pressure shocks (Yamazaki and Goodier, 1993). Desirable values of pressure are of the order of 470 to 700 atmospheres, dispensed as a quick shock, usually 4 to 10 min long. Several commercial apparatuses are available for the induction of polyploidy in fish by pressure shocks. In most cases, pressure is built up by forcing water from a reservoir into a pressurised container where the eggs are kept. The rapidity on reaching the desired pressure seems to be of primary

importance for obtaining a good percentage of triploid individuals (Benfey *et al.*, 1984).

1.5.2 Indirect methods

Triploid fish have been produced by indirect methods, which are based on the expectancy of the production of diploid gametes by tetraploid individuals. Fertilisation of normal (haploid) ova by diploid sperm results in the generation of a triploid offspring. This approach is of particular interest for the generation of triploids in species where the use of direct methods is impaired by low fecundity and difficulties for artificial spawning such as territorial catfishes or mouth-breeding cichlids (Chourrout, 1984; Hussain, 1996). Although it was initially suggested that tetraploidy might be difficult to induce or it may result in inviable chromosomal arrangements (Purdom, 1983), the generation of tetraploid broodfish has been successfully accomplished in salmonids (Thorgaard *et al.*, 1981; Chourrout, 1984; Chourrout *et al.*, 1986; Diter *et al.*, 1988). Chourrout *et al.* (1986) obtained fertile tetraploid male rainbow trout and successfully bred tetraploid male and diploid females to produce viable triploids. Tetraploids have also been produced in *Oreochromis* spp. (Valenti, 1975; Myers, 1986; Pandian and Varadaraj, 1987; Don and Avtalion, 1988b) and other teleost species such as the channel catfish (Bidwell *et al.*, 1985), but the poor viability and reproductive performance of these individuals limits the commercial applicability of this methodology (Thorgaard, 1986; Hussain, 1996).

1.6 Methodology for ploidy evaluation

Techniques for triploidy induction are never totally effective. During the generation of triploid fish, some non-triploid individuals are inadvertently produced, and in order to eliminate undesired spawning of diploid forms only triploids should be stocked. Thus, presumptive triploid fish generated by the techniques described in the previous section must be screened and their triploid condition must be individually confirmed. Ideal features for the methodology of ploidy evaluation include accuracy, rapidity and cost-effectiveness; to be practical for the fish farming industry, these techniques must also be able to analyse large numbers of fish (Ihssen *et al.*, 1990; Mair, 1993). The techniques that have been developed for ploidy assessment in fish can be classified in three categories: cytogenetic methods, the measurement of erythrocyte nuclear or whole-cell size and the measurement of nuclear DNA content.

1.6.1 Cytogenetic methods

Cytogenetic methods include the counting of chromosomes and nucleoli. The term *karyotype* usually refers to the arrangement of metaphase chromosomes in a sequence according to their length and position of the centromere. Karyotyping has revealed the existence of triploid individuals in natural and domesticated populations of teleosts (Cuéllar and Uyeno, 1972; Thorgaard and Gall, 1979). Chromosome preparation and counting is inexpensive and requires little specialised equipment; although several standardised methods have been described, the best results are obtained with preparations from embryos or very young fish, unless cell culture is used (McPhail and Jones, 1966; Denton and

Howell, 1969; Gold, 1974; Kligerman and Bloom, 1977; Hartley and Horne, 1983; Thorgaard and Disney, 1990). The production of karyotypes still remains the only method for determining precise chromosome numbers and configuration, but it is a laborious process that cannot be used in the practical screening of presumptive-triploid fish populations.

A more simplistic approach for the cytogenetic evaluation of fish ploidy is the counting of nucleoli after silver staining (Phillips *et al.*, 1986). This is a simple and reliable technique, but an adequate cytogenetic background is required to correlate ploidy levels with the number of nucleolar-organising regions present in the karyotype (Foresti *et al.*, 1981; Phillips and Ihssen, 1985; Iturra *et al.*, 1990).

1.6.2 Determination of erythrocyte nuclear or whole-cell size

Nuclear size analyses are based on the increase in the nuclear volumes resulting from the higher amount of DNA and number of chromosomes present in triploid cells. The relationship between the size of the nuclei and the size of the cytoplasm makes the reliable assessment of ploidy by whole red blood cell analysis possible. Unlike mammals, fish red blood cells are nucleated which, added to their consistent size and ease of sampling, facilitates the use of these techniques in teleosts (Cimino, 1973; Benfey *et al.*, 1984). Although the microscopic measurement of stained erythrocyte nuclei is simple and relatively inexpensive, shortcomings of these techniques include the time delay associated with fixing, staining and analysing the slides or smears.

The possibility of automating the process by electronically analysing erythrocyte nuclear volumes was investigated by Johnson *et al.* (1984) who reported that a comparison between ploidy analyses performed by measuring

erythrocyte nuclei with an ocular micrometer and electronically with a particle counter (Coulter Counter) resulted in complete agreement. A Coulter Counter operates by passing particles (nuclei or whole red blood cells) suspended in an electrolyte solution through an appropriately sized orifice and detecting the resulting change in resistance which is proportional to particle size. The Coulter Counter provides a rapid and efficient method for estimating ploidy in fish, and large numbers of individuals can be accurately analysed. Relatively high operating costs and the need for specialised training restrict the use of this technique to large-scale fish farming operations (Johnson *et al.*, 1984; Bye and Lincoln, 1986; Ihssen *et al.*, 1990).

1.6.3 Quantification of DNA

The methods based on the colourimetric or fluorimetric quantification of deoxyribonucleic acid (DNA) reveal nuclear DNA with specific-affinity stains (DAPI, Feulgen stain, propidium iodide)(Allen *et al.*, 1983; Teplitz *et al.*, 1990). Flow cytometry is used to rapidly analyse the DNA content of large numbers of interphase cells with accuracy exceeding that of other quantitative techniques (Allen, 1983; Johnson *et al.*, 1984; Benfey *et al.*, 1986). Very small blood samples can be analysed, and somatic tissues may also be processed after nuclear isolation. The elevated cost of the apparatuses involved and the specialised technical knowledge required limit the use of these methods at the farm level.

1.7 Gametogenesis

Gametogenesis is a process of cellular differentiation that results in the transformation of undifferentiated gonial cells (spermatogonia or oogonia) into fertilisable gametes. *Spermatogenesis*, or the generation of spermatozoa, takes place in the male gonad, the testis. The process of gametogenesis in females results in the generation of ova and receives the name of *oogenesis*. Gametogenesis is preceded by the differentiation of *primordial germ cells* into the male or female direction (Kinne, 1991). A review of spermatogenesis and oogenesis in the rainbow trout with special emphasis on the cellular classes observed during these processes is presented here with the aim of providing general background for the studies on gametogenesis included in the present Thesis. The correspondence between meiotic stages and the cellular classes observed during the spermatogenesis and oogenesis in the rainbow trout is summarised in Table 1.1.

<u>Spermatogenesis</u>	<u>Meiotic stages</u>		<u>Oogenesis</u>
Spermatogonia	Pre-meiotic Interphase		Oogonia
Primary Spermatocyte	Prophase I	First Meiotic Division	Stage 1 Oocytes
	<i>Leptotene</i>		Stage 2 Oocytes
	<i>Zygotene</i>		Stage 3 Oocytes
	<i>Pachytene</i>		
	<i>Diplotene</i>		
	<i>Diakinesis</i>		Stage 4 Oocytes
	Metaphase I		Stage 5 Oocytes
	Anaphase I		Stage 6 Oocytes
	Telophase I		Stage 7 Oocytes
Secondary Spermatocyte	Interphase	Second Meiotic Division	Ova
	Prophase II		
	Metaphase II		
	Anaphase II		
	Telophase II		
Spermatid			
Spermatozoa			

Table 1.1 Cellular classes and corresponding meiotic stages observed during the spermatogenesis and oogenesis of the rainbow trout (after Billard, 1986, 1992; Bromage and Cumaranatunga, 1988).

1.7.1 Sex differentiation and puberty

In the rainbow trout, testes and ovaries are paired organs located in the body cavity. The anterior end of the gonad is attached to the antero-dorsal area of the body cavity in which it is located, and during early stages of gonadal differentiation, some connections exist between the gonad and the mesonephros with possible cell migration between both organs (Billard, 1992). Signs of sex differentiation appear first in females between 18 and 28 days post-hatching at an incubation temperature of 11.5 °C (Van der Hurk and Sloff, 1981). Takashima *et al.* (1980) reported similar findings, with occurrence of sex differentiation shortly after completion of yolk sac resorption, 2 months after fertilisation. Lebrun *et al.* (1982) found that the gonads in the rainbow trout remain undifferentiated for 4 weeks post-hatching (p.h.) at 10-11°C; at 35 days p.h. some enlargements of the anterior part of the gonad occur. At 42 days p.h. the first sign of ovarian organisation of the gonads could be identified. The formation of the ovarian lamellae and the completion of the structural development of the ovary occur at 12-16 weeks of age (Billard, 1992). Changes in the number of primordial germ cells in the gonads were also reported by Lebrun *et al.* (1982); immediately after hatching the average number of germ cells is low (around 50 in female gonads) and slowly increases to 2,000 at 5 weeks p.h. After 5 weeks, signs of meiosis can be observed in some of the anteriorly-enlarged gonads, with a sharp increase in the number of germ cells, resulting in the identification of up to 11,000 germ cells at 10 weeks p.h., when the ovary consists mostly of meiotic oocytes (Lebrun *et al.*, 1982). In the filament-like gonadal type, which is assumed to represent an

undifferentiated testis, the germ cells remain undifferentiated and are fewer in number (Billard, 1992).

During the first meiotic prophase, the synthesis of nuclear DNA is slow through the leptotene and zygotene stages, rapid in early pachytene and absent in early diplotene (Vlad, 1976). The capacity to synthesise or metabolise sex steroids exists in still morphologically undifferentiated gonads at day 23 post-hatching for progestins, at day 73 p.h. for androgens in the testis, and at day 173 p.h. for oestrogens in ovaries (Van der Hurk *et al.*, 1982). Van der Hurk and Oordt (1985) have shown that 11-oxygenated androstendione derivatives, possibly synthesised from non-gonadal corticosteroids, play an important role in sustaining the differentiation and early development of the testis. Sex differentiation appears labile at these early stages of development and sex inversion can easily be obtained by feeding the fry with food containing methyl-testosterone or oestradiol (Jalabert *et al.*, 1975; Hunter and Donaldson, 1983).

It has been recently shown that steelhead trout embryos are able to form steroid glucuronides during very early life stages (Yeoh, 1996a, b). Steroid levels are relatively high at 1 day post-fertilisation in rainbow trout embryos and decline until 25 days post-fertilisation (Feist and Schreck, 1996). At hatching, steroid levels increase slightly before they fall by 78 days post-fertilisation and remain relatively constant thereafter. Trends toward differences in steroid content between female and male monosex populations of rainbow trout become evident around the time of morphological (based on histology) gonadal differentiation (78-90 days post-fertilisation; Feist and Schreck, 1996). GTH 1 and GnRH can be detected in rainbow trout pituitary and brain as early as 48 days after fertilisation (Feist and

Schreck, 1996). The activity detected in the brain-pituitary-gonadal axis during the process of sex differentiation, together with the fluctuations in steroid hormones observed at this time, have led to the suggestion that sex steroids may play a driving role for sexual differentiation in the rainbow trout (Feist and Schreck, 1996; Yeoh *et al.*, 1996a, b).

1.7.2 Organisation of the testes in the rainbow trout

Rainbow trout develop testes of the lobular type (Billard, 1986). In this type of teleostean testicular organisation, connective tissue extends from the testicular capsule to form irregular tubules lined with a Sertoli cell epithelium including germ cells (Figure 1.2, A). The blind end of the tubules is apposed against the testicular capsule; the tubules converge ventrally towards a sperm collection system or *sperm duct*. In the lobular structure early stages of spermatogonia are found along the entire length of the lobule. Later stages of spermatogonia and spermatogenic cells appear in cysts which are groups of germ cells dividing synchronously and surrounded by Sertoli cells (Figure 1.2, B). Spermatozoa are released into the lobule lumen from which they reach the efferent and defferent systems (Grier, 1981; Billard, 1986).

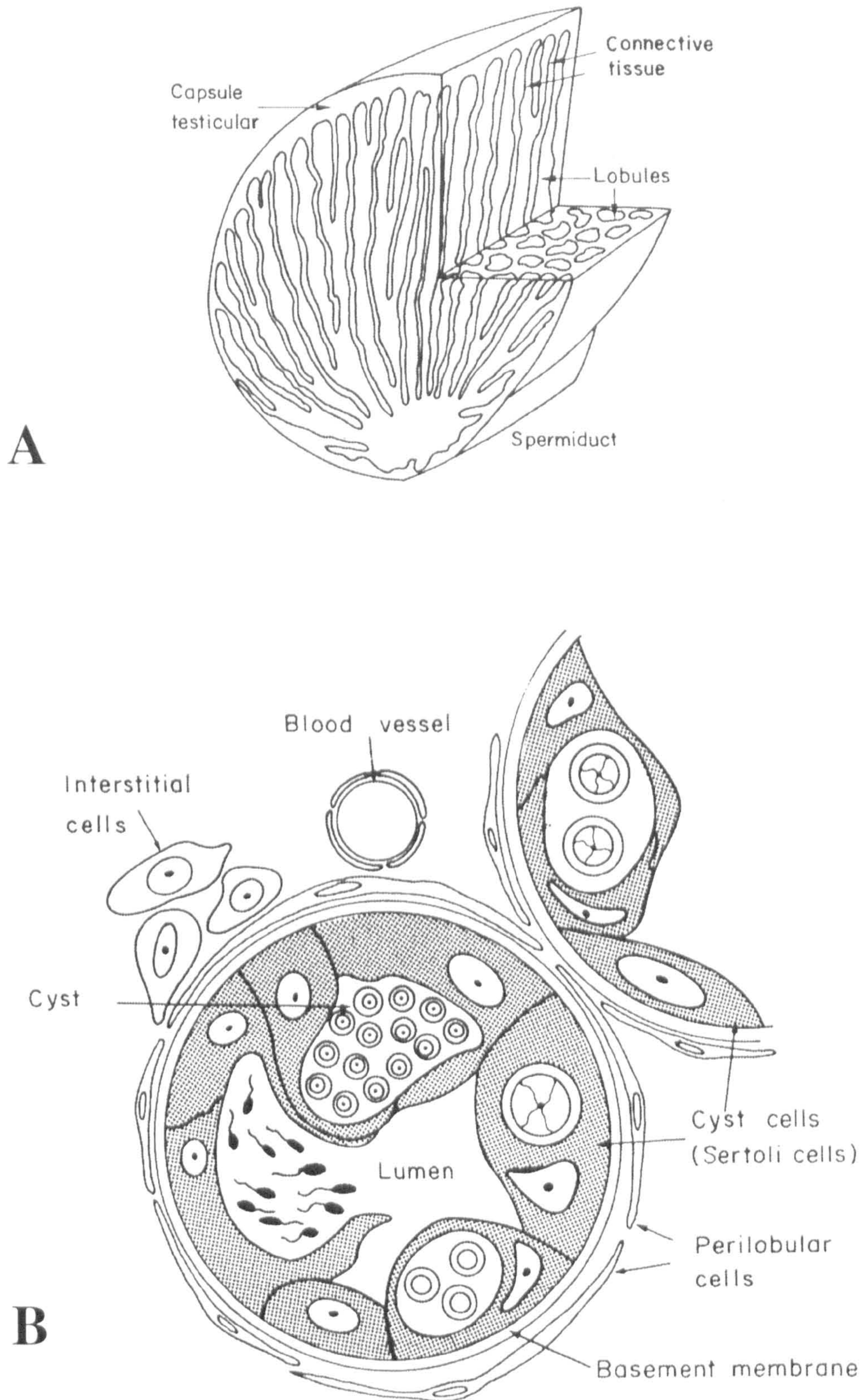


Figure 1.2

A, Organisation of the testes in the rainbow trout (Billard, 1986)

B, Schematic representation of the internal structure of a testicular lobule and the interlobular space in the rainbow trout (Billard, 1992)

1.7.3 Gonadal cell stages in males

1.7.3.1 Spermatogonia

Two types of spermatogonia have been identified on the basis of morphological criteria in the rainbow trout. *Type A spermatogonia* are characterised by their large nuclei (8-10 μm in diameter) with one large, centrally-positioned nucleolus and several chromatin filaments radiating towards the periphery of the cytoplasm. They have few structured organelles, scattered mitochondria and little reticulum, and frequently show mitotic activity. *Type B Spermatogonia* are grouped in cysts in the rainbow trout and seem to be irreversibly engaged in spermatogenesis. They are characterised by smaller nuclei than type A spermatogonia (3-4 μm in diameter), more dense chromatin and a higher number and diversity of cellular organelles (Billard, 1986; Kinne, 1991).

1.7.3.2 Spermatocytes

Primary spermatocytes are distinguished from spermatogonia type B by their larger nuclei (4-6 μm) and by the resulting increase of the size of the cysts where they are contained. They are round cells with darkly-staining nuclei centrally positioned and scarce cytoplasm. Primary spermatocytes within a cyst are connected by cytoplasmic junctions which ensure the synchronous progress of these cells through the various phases of the first meiotic division; these junctions persist until the spermatid stage (Billard, 1983). *Secondary spermatocytes* are smaller in size and usually appear in a more central position within the cysts than primary spermatocytes. Their centrally-positioned nuclei are approximately 2-3 μm in

diameter, stain intensely dark with haematoxylin-eosin and have an spheroidal shape with irregular contours.

1.7.3.3 *Spermatids and spermatozoa*

Ultrastructural observations in the rainbow trout have identified four different *spermatid* sub-stages (Billard, 1983). Under the light microscope, spermatids appear as small (3-4 μm in diameter), round cells with a large, darkly-staining nucleus and very scarce cytoplasm, generally occupying a central position within the cysts. Their nucleus elongates slightly as the chromatin becomes more condensed during the transition spermatid-spermatozoa. *Spermatozoa* in the rainbow trout are characterised by their elongated nuclei and well-developed mid-piece, and lack an acrosome. Under the light microscope, they appear as round, darkly-staining spheroidal cells with densely packed chromatin and no distinctive cytoplasm, filling the central spaces of the testicular lobules in mature males (Billard, 1986; Kinne, 1991).

1.7.3.4 *Sertoli cells*

Sertoli cells are elongated, pyramidal cells with cytoplasmic projections that almost entirely surround the cells of the spermatogenic lineage (Figure 1.1, B)(Grier, 1981; Billard, 1986). Sertoli cells contain numerous lipid droplets in their cytoplasm, but under the light microscope their outlines appear poorly delimited because of the numerous lateral processes that surround spermatogenic cells (Grier *et al.*, 1981). Unlike other teleosts, the population of Sertoli cells appears to be permanent in the rainbow trout, and among the several roles attributed to them

are (a), the support and structuration of the lobes and cysts; (b), the transfer and conversion of metabolites and hormones towards the spermatogenic cells; (c), the phagocytosis of germ cells and residual spermatozoa, and (d), the isolation of the cyst compartment beyond the spermatocyte stage (Billard, 1986; Kinne, 1991).

1.7.3.5 *Interstitial cells and the interlobular space*

The connective tissue in the testicular capsule of the rainbow trout is composed of *fibroblasts* bordered on the lobule side by a basal lamina against which Sertoli cells are apposed (Figure 1.1, B)(Billard, 1986). The fibroblasts form a discontinuous layer around the lobules. Other components of the interlobular space are *myoid* or *perilobular cells*, which possess ultrastructural characteristics similar to boundary cells in the mammalian testis (Grier, 1981). Ultrastructurally, the teleost myoid cell cytoplasm contains numerous microfilaments oriented in parallel to the long axis of the cell, few to numerous pynocytotic vesicles, an elongated nucleus and rough endoplasmic reticulum (Grier, 1981).

Using morphological criteria, *interstitial* cells can be distinguished between the fibroblast layers. Interstitial cells appear to be homologous to mammalian Leydig cells in most teleosts, characterised by a fusiform or polyhedral shape, a central nucleus and an eosinophilic cytoplasm rich in small lipid droplets (Grier, 1981; Van der Hurk *et al.*, 1982). Interstitial cells are involved in steroidogenesis, as indicated by their positive staining for 3 β -hydroxysteroid dehydrogenase (Van der Hurk and Slof, 1981; Van der Hurk *et al.*, 1982). In the rainbow trout, interstitial cells participate in the synthesis of 11-ketotestosterone (Van der Hurk *et al.*, 1978; Kinne, 1991; Nagahama, 1994).

The rainbow trout testis appears to lack a lymphatic system as described in mammals, but it does possess an extravascular space containing blood plasma which leaks between blood vessel *endothelial* cells (Grier, 1981).

1.7.4 Spermatogenesis, spermiogenesis and spermiation in the rainbow trout

The process of spermatogenesis is not synchronous in the rainbow trout: at the initiation of spermatogenesis in May-June some cells of the spermatogenic lineage reach the stage of spermatids, so that spermatozoa can be occasionally found in the lobular lumen before the size of the testis has markedly increased. The bulk of spermatogenic activity occurs later in July-August as shown by the change in weight of the testis as percent of the body weight, or *gonado-somatic index*. Intense activity can be observed at that time in the Sertoli and Leydig cells, with changes in their structure and steroidogenic activity (Loir, 1990).

During *spermiogenesis*, or the differentiation of spermatids into spermatozoa, the majority of the cytoplasm of the spermatids is eliminated and phagocytosed by the Sertoli cells. Only some remnants of cytoplasm and a few mitochondria are found in mature spermatozoa (Billard, 1983). At the end of spermiogenesis the spermatozoa are released from the cysts into the lobular lumen. Progressively the lumen becomes filled with spermatozoa and no other germ cell stages are present except type A spermatogonia, which will be the starting point of the next spermatogenic cycle. Spermatozoa remain in the lobule for approximately 1 month in an apparently quiescent state, where they may undergo some final maturation (Billard, 1983, 1992).

In the rainbow trout *spermiation* corresponds to the release of spermatozoa from the lumen of the lobules into the sperm duct, probably after a rise in hydrostatic pressure inside the lobule due to the secretion of fluid by the Sertoli cells under gonadotropic stimulation (Billard, 1992). Spermatozoa are then pushed into the efferent and defferent systems that are also secretory and contribute to the fluidity of the milt. The sperm duct has two parts: one is adjacent to the testis and collects spermatozoa at the opening of the lobule (*juxta-testicular part*); the other is a simple duct connecting the posterior part of the testis to the genital papilla (Figure 1.2, A)(Billard, 1992). There are no seminal vesicles in the rainbow trout. The free part of the sperm duct seems to have several functions: (a), regulation of the ionic composition of the seminal fluid (Morisawa and Morisawa, 1990); (b), resorption of spermatozoa (Billard and Takashima, 1983), and (c), the metabolism and/or secretion of hormones (Van der Hurk *et al.*, 1978; Schulz, 1986).

1.7.5 Ovarian structure in the rainbow trout

Rainbow trout develop ovaries of the *gynovarian* type (Hoar, 1969; Guraya, 1986). In this type of teleostean ovary, the peritoneum covers the outer surface of the ovary only partially, and ova are directly discharged into the peritoneal cavity. The ovaries are supported in the body cavity by a dorsolateral fold of the peritoneum. A branch of the dorsal aorta and the ovarian vein run parallel to the long axis of the ovary along the margin where the peritoneal membrane comes in contact with the ovary. Arterial and venous supplies individually serve each developing follicle, with blood from the ovarian vein draining directly into the Cuvierian sinus (Bromage and Cumaranatunga, 1988). The outer wall of the ovary, continuous with the peritoneum, is referred to as *tunica albuginea*. The tunica albuginea contains smooth muscle cells and densely packed collagen, elastin and reticulin fibres, it is highly vascularised, and it is externally lined by a squamous to cuboidal epithelium (Van der Hurk and Peute, 1979).

Connective tissue septae known as *trabeculae* extend perpendicularly from the tunica albuginea into the ovarian stroma. The connective trabeculae are organised transversally in a series of folds extending along the ovarian axis which receive the name of *ovigerous lamellae*. The inner epithelium covering the lamellae is referred to as *germinal epithelium* since female germ cells develop in association with this structure.

The ovary of the rainbow trout contains germ cells that develop synchronously; at least two developmental phases of oocytes are observed during the process of oogenesis and females spawn once a year for a number of years

(Bromage and Cumaranatunga, 1988). Similarly to other teleosts, seven stages of oocyte development have been identified in the rainbow trout on a morphological basis.

1.7.6 Gonadal cell stages in females

1.7.6.1 Stage 0 (Oogonia)

Oogonia are large cells (12-17 μm in diameter) morphologically characterised by a large nucleus containing one or more small nucleoli and an indistinct band of cytoplasm (Figure 1.3)(Browder, 1985; Bromage and Cumaranatunga, 1988). In teleosts and amphibians, unlike in other vertebrates, dividing oogonia persist in the adult ovary (Billard, 1987; Selman *et al.*, 1993; Tyler and Sumpter, 1996) and they may continue to divide throughout the life cycle (Tokarz, 1978). Oogonia in the rainbow trout may appear isolated or forming clusters or nests, where they tend to be slightly larger in size and with a more round cytoplasm, frequently showing mitotic activity (Takashima *et al.*, 1980). This different arrangement of oogonia in the rainbow trout is the basis for the distinction between *primary* and *secondary oogonia*, similarly to what is observed in males. Morphological differences between the two types of oogonia observed in the rainbow trout are not well documented, and thus in the present study primary and secondary oogonia will be described together. Oogonial cysts are frequently surrounded by smaller, flattened cells with a fusiform nucleus known as *prefollicle cells*, which have been described as having a mesenchymal or epithelial origin (Tyler and Sumpter, 1996). During the differentiation of the follicle, prefollicle cells

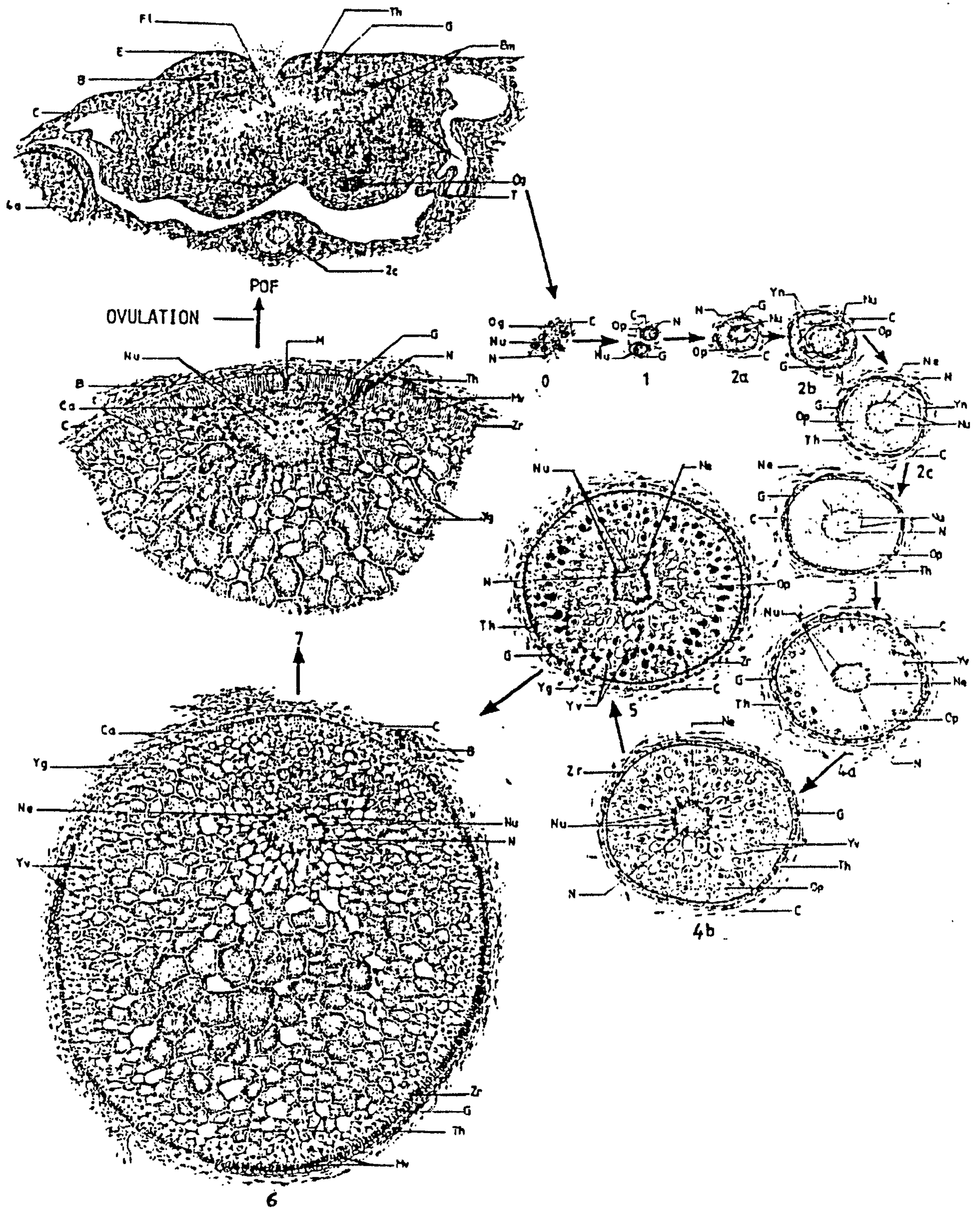


Figure 1.3. Oocyte developmental cycle of the rainbow trout. Key: O, oogonia; 1-7, oocytes at stage 1 to 7; POF, post-ovulatory follicle; B, blood vessels; Bm, basement membrane; C, connective tissue; Ca, cortical alveoli; E, epithelium; Fl, follicle lumen; G, granulosa; M, micropyle; Mv, microvilli; N, nucleus; Ne, nuclear envelope; Nu, nucleoli; Og, oogonia; Op, ooplasm; T, trabeculae; Th, theca; Yg, yolk granules; Yn, yolk nuclei; Yv, yolk vesicles; Zr, zona radiata. (Bromage and Cumaranatunga, 1988).

become increasingly flattened and migrate to form a cell layer around individual oocytes (Bromage and Cumaranatunga, 1988; Tyler and Sumpter, 1996).

1.7.6.2 Stage 1 Oocytes (*Chromatin nucleolar stage*)

Stage 1 oocytes are slightly larger than oogonia, with diameters ranging from 24 to 46 μm (Bromage and Cumaranatunga, 1988). Their nuclei contain several strands of basophilic chromatin positioned under the nuclear membrane, occupying a major portion of the oocyte with only a narrow encircling band of slightly basophilic cytoplasm (Figure 1.3). Oocytes at this stage have differentiated a primordial *follicle*, formed by the association of stage 1 oocytes with a surrounding monolayer of 3-4 flattened *granulosa* cells (Browder, 1985; Bromage and Cumaranatunga, 1988; Tyler and Sumpter, 1996).

1.7.6.3 Stage 2 Oocytes (*Balbani body stage or Early perinucleolar stage*)

Stage 2 oocyte diameter ranges from 80 to 240 μm (Bromage and Cumaranatunga, 1988). Their nuclei contain one large, spherical nucleolus and several smaller peripheral nucleoli (Figure 1.3). At this stage of oocyte development the basophilic nature of the cytoplasm reaches a maximum while the nuclear basophilia gradually diminishes. Stage 2 oocytes are characterised by the appearance of shallow undulations in the nuclear envelope accompanied by the presence of basophilic electron-dense deposits in the juxtannuclear region termed *Balbani bodies*. Balbani bodies are composed of ribonuclear proteins associated to hetero-nuclear mRNA transported from the nucleus into the cytoplasm through nuclear membrane pores (Olins *et al.*, 1993; Visa *et al.*, 1996). Stage 2 oocytes are surrounded by a single squamous granulosa cell layer and one or two squamous

thecal cell layers (Browder, 1985). Thecal and granulosa cells play a critical role in the metabolism of sexual steroids and the nourishment of the growing oocyte (Kagawa *et al.*, 1982; Nagahama, 1994).

1.7.6.4 Stage 3 Oocytes (*Late perinucleolar stage*)

The late perinucleolar stage is characterised by the presence of small spheroidal nucleoli situated closely to the nuclear membrane. During this stage the basophilic nature of the cytoplasm becomes reduced and the Balbiani bodies migrate to the cellular periphery (Van der Hurk and Peute, 1979; Takashima *et al.*, 1980). Stage 3 oocytes increase in size, reaching diameters ranging from 300 to 400 μm , and by the end of the late perinucleolar stage the follicle consists of a growing *zona radiata*, a granulosa layer and an outer double celled theca layer (Bromage and Cumaranatunga, 1988; Kinne, 1991)(Figure 1.3). The *zona radiata* or *vitelline envelope* appears under the light microscope as a ring composed of a number of radial striations between the granulosa cells and the oocyte; it is formed by the proliferation of microvilli from the cellular membrane of the oocyte and the deposition of two to four major proteins synthesised by the liver under the influence of oestrogen (Bromage and Cumaranatunga, 1988; Hyllner *et al.*, 1991, 1994).

1.7.6.5 Stage 4 Oocytes (*Vesicle stage*)

During this stage PAS-positive, glycoprotein-rich vesicles termed *cortical alveoli* accumulate initially in the periphery of the cytoplasm, and subsequently in the entire cytoplasm of the oocyte, resulting in a reticulate appearance under the light microscope (Figure 1.3). These vesicles contain a polysialoglycoprotein

complex of at least 200 kilodaltons of molecular weight endogenously synthesised by the oocyte (Guraya, 1986; Inoue and Inoue, 1986; Ho, 1991; Hyllner *et al.*, 1994).

1.7.6.6 Stage 5 Oocytes (*Peripheral yolk granule stage*)

Stage 5 oocytes are easily distinguished from the previous stage by the presence of small exogenous yolk-rich granules (*yolk granules*) in the cytoplasmic region adjacent to the zona radiata, staining pink with haematoxylin-eosin (Guraya, 1986; Bromage and Cumaranatunga, 1988). These yolk granules coalesce to form larger yolk globules positioned in the centre of the oocyte at later stages of development. Stage 5 oocytes contain a central nucleus and are surrounded by an enlarged zona radiata, a well developed granulosa and a theca which ultrastructurally contains an abundant rough endoplasmic reticulum (Figure 1.3)(Kinne, 1991).

1.7.6.7 Stage 6 Oocytes (*Migrating germinal vesicle stage*)

This oocyte stage is characterised by a migrating nucleus or *germinal vesicle*, which appears in an eccentric position within the cytoplasm and with small peripheral nucleoli (Guraya, 1986; Bromage and Cumaranatunga, 1988). Abundant yolk aggregations are evident in the cytoplasm of stage 6 oocytes, with large yolk globules in the vegetal pole opposed to the germinal vesicle developing into polygonal *yolk platelets* by late stage 6. The follicle at this stage contains a thick zona radiata and a granulosa composed of flattened granulosa cells (Figure 1.3)(Van der Hurk and Peute, 1979; Guraya, 1986). Although the migration of the germinal vesicle has been shown to coincide with an increase in oocyte sensitivity

to 17 α -hydroxy-20 β dihydro-progesterone and maturational gonadotropin (Moley and Schreiber, 1995), the precise mechanism that results in the displacement of the nucleus has yet to be elucidated (Hart and Fluck, 1995).

1.7.6.8 Stage 7 Oocytes (*Germinal vesicle break-down stage*)

Stage 7 oocytes are characterised by the rupture of the nuclear membrane, a process known as *germinal vesicle break-down*. These oocytes appear as large, yolk-filled structures with peripherally-located and ruptured nuclear envelopes (Bromage and Cumaranatunga, 1988; Moley and Schreiber, 1995). The follicles contain a large zona radiata with prominent radial striations; within the zona radiata and immediately opposite to the germinal vesicle a micropyle is sometimes visible, covered by enlarged granulosa cells (Figure 1.3)(Bromage and Cumaranatunga, 1988).

1.7.6.9 Post-ovulatory follicles

Post-ovulatory follicles are hollow collapsed structures morphologically characterised by the presence of a hypertrophied granulosa and a theca which is continuous with the epithelial cell lining of the ovigerous lamellae (Figure 1.3). These structures result from the ovulation of mature stage 7 oocytes, which are forced out of the follicles leaving behind the granulosa and thecal layers. Oogonial nests are often found among the connective tissue and ovigerous lamellae surrounding the post-ovulatory follicles; it is from these that further cohorts of pre-vitellogenic oocytes arise (Bromage and Cumaranatunga, 1988).

1.7.6.10 Atretic oocytes

Atresia is a degenerative process by which oocytes are resorbed before the completion of maturation. Atresia may affect different stages of oocyte development, but most studies on its incidence have been limited to oocytes in late stages of development (stage 4 onwards); very little is known about atresia in early (1-3) stages of oocyte development (Bromage and Cumaranatunga, 1988; Moley and Schreiber, 1995; Tyler and Sumpter, 1996). Histologically, the first sign of atresia is the shrinkage of the follicle, which may involve separation of the different follicular layers from the oocyte. Subsequently, atresia assumes a variety of forms depending on the oocyte stage which is being resorbed and also on the stage of development of the ovary. During early stages of atresia the nucleus of the oocyte ruptures discharging its contents into the cytoplasm; in advanced stages of oocyte development the yolk becomes more liquefied and vacuolated, losing acidophilia. Characteristically, the granulosa layer becomes hypertrophied, penetrating the zona radiata and phagocytising the contents of the oocyte cytoplasm (Bromage and Cumaranatunga, 1988).

There is some controversy in the current literature about the incidence and physiological role of atresia in teleostean gametogenesis. In the rainbow trout, reduced feeding results in a progressive increase in the incidence of atresia, and complete starvation may result in resorption of the full complement of maturing oocytes (Bromage and Cumaranatunga, 1988). Quantification of the degree of atresia is difficult; it is possible that some of the incidences of atresia reported in studies using non-virgin females are due to degenerating oocytes from a previous sexual cycle, which cannot be distinguished from oocytes maturing at the time of

the analysis on the basis of size alone (Tyler *et al.*, 1990, 1994). In spite of recent interest in the atretic process, the factors which bring about its occurrence and the dynamics of the resorptive process have not yet been fully clarified (Moley and Schreiber, 1995; Tyler and Sumpter, 1996).

1.7.7 Oogenesis and ovulation in the rainbow trout

The ovarian lamellae start organising at 5 weeks post-hatching in the rainbow trout, when they contain an inner core of mesenchymal stroma, fibroblasts and interstitial cells covered by a multilayered epithelium in which the oogonia are initially embedded (Upadhyay, 1977). At this stage of development, oogonia are organised into clusters and by 12-16 weeks post-hatching they become progressively isolated by pre-follicular cells and included in the mesenchymal stroma (Upadhyay, 1977; Billard, 1992). Once incorporated in the stroma, oocytes undergo a complex process of growth involving a massive incorporation of yolk into their cytoplasm. It takes at least 6 months for the growth process to be fully complete; this may explain the later response of the ovary to exogenous GTH stimulation, compared to the testis (Billard, 1992). The basic pattern of oocyte growth in the rainbow trout is common to all teleosts, and can be generally divided in four different phases: primary growth, vesicle or cortical alveolus stage, vitellogenesis and maturation (Wallace and Selman, 1981).

The *primary growth* of the oocytes (oocyte stages 0-3) is GTH-independent and it is characterised by a complex accumulation of RNA (5s RNA and transfer RNA) and ribonucleoproteins which are generally responsible for the strong basophilia observed in early oocyte stages. RNA is subsequently located in storage

particles before being transferred for ribosomal translation (Guraya, 1986; Olins *et al.*, 1994; Visa *et al.*, 1996).

The *vesicle stage* (oocyte stage 4) is considered to be the first GTH-dependent stage. During this phase of oocyte growth the formation of vesicles that eventually will form the cortical alveoli takes place. These cortical alveoli play an important role in the cortical reaction induced by osmotic shock at the time of fertilisation. It is also during this phase that the zona radiata starts to form. The zona radiata will become the future chorion of the hardened egg (Nosek, 1984; Bromage and Cumaranatunga, 1988, Tyler and Sumpter, 1996).

The phase of *vitellogenesis* includes oocyte stages 5 and 6, and it is characterised by a dramatic increase in their cytoplasmic volume as a result of the incorporation of vitellogenin and other extraovarian proteins. Vitellogenin is a lipophosphoprotein-calcium complex synthesised by the liver in response to circulating oestradiol-17 β derived from the ovary (Tyler and Sumpter, 1996). The blood vitellogenin is taken up by the oocyte in a receptor-mediated process and deposited in coated vesicles in the oocyte cytoplasm (Ho, 1991; Tyler and Lancaster, 1993; Hyllner *et al.*, 1994; La Fleur *et al.*, 1995). The process of vitellogenin deposition within the oocytes is accompanied by the displacement of the nucleus or germinal vesicle towards the periphery of the cell (Hart and Fluck, 1995).

The final migration of the germinal vesicle, which eventually comes in contact with the oocyte membrane in close proximity to the micropyle, and the rupture of the nuclear envelope define the *maturation* phase. Ovulation occurs shortly after oocyte maturation, it is controlled by hormonal influences and results

from the rupture of the follicular wall (Tyler and Sumpter, 1996). At the same time the micropylar cell that blocks the micropyle is removed so that spermatozoa can penetrate the chorion after oviposition. In the rainbow trout, immediately prior to ovulation, the glycoproteins that accumulate in the cortical alveoli undergo depolymerisation into smaller 9 kilodaltons fragments (Inoue and Inoue, 1986), and occupy a position between the zona radiata or chorion and the egg cytoplasm. It is into this area, the *peri-vitelline space*, that water is taken after fertilisation (i.e. during water hardening). The contents of the cortical alveoli serve to harden the chorion of the egg, rendering it impervious to further movement of water and preventing polyspermy (Bromage and Cumaranatunga, 1988; Kinne, 1991; Kitajima *et al.*, 1994; Moley and Schreiber, 1995).

1.8 Structure and aims of this Thesis

The present Thesis consists of one general Chapter covering the Materials and Methods of general relevance for the experimental work (Chapter II), and four experimental chapters (Chapters III to VI), in addition to this General Introductory Chapter.

The species selected for the experiments described in Chapters III to V is the rainbow trout, *Oncorhynchus mykiss* (n=58 to 64, NF= 104, Ohno *et al.*, 1965; Thorgaard, 1976; Hartley and Horne, 1982; Veloso *et al.*, 1990). The rainbow trout is at present the mainstay of large-scale salmonid aquaculture on a world wide basis (Pillay, 1993). The increasing sophistication of the retail market for trout is demanding a continuity of product both in terms of the quality and size of fish produced, as well as the maintenance of regular supplies throughout the year (Bromage and Cumaranatunga, 1988). The accomplishment of these demands requires both knowledge and control of all aspects of the biology of this species, of which reproduction is possibly the most important. Deteriorative changes in the organoleptic characteristics of the flesh, together with increased mortalities and susceptibility to disease, particularly in males, have prompted the development of methods aimed to delay or prevent sexual maturation in this species (Thorgaard, 1986; Bromage and Cumaranatunga, 1988; Hussain, 1996). The induction of triploidy stands today as a cost-efficient method for the control of the deleterious effects that sexual maturation brings about in farmed stocks of rainbow trout; hence the selection of this species for the present Thesis.

The current literature on the general performance of triploid teleosts, and in particular the salmonids, provides conflicting evidence on the yield of triploids for

commercially-relevant traits such as growth, weight gain or survival (Ihssen *et al.*, 1991; Myers and Hershberger, 1991; Simon *et al.*, 1993; Carter *et al.*, 1994; Galbreath and Thorgaard, 1995). Chapter III is therefore intended to assess the general performance of autotriploid rainbow trout under controlled culture conditions, with special attention on yield indicators of particular commercial significance (e.g. weight of the eviscerated carcass).

Chapter IV focuses on the process of gonadal development and gametogenesis in male and female autotriploid rainbow trout from early to late life stages. Although the gonadal development of triploid rainbow trout has been the subject of previous investigations (Lincoln and Scott, 1984; Nakamura *et al.*, 1987, 1993; Kobayashi *et al.*, 1993; Cerisola and Dazarola, 1996), the majority of these reports have focused on the histomorphology and reproductive endocrinology of triploid forms at the time of sexual maturation. Chapter IV was thus designed to provide a long-term view of the gametogenic process in triploid rainbow trout, with the specific goal of quantifying the degree of germ line development in triploid forms of this species, particularly in the light of recent findings on the gonadal development of triploid poultry (Lin *et al.*, 1995a, b).

A number of adult males in the triploid population of rainbow trout generated for the studies included in the present Thesis displayed secondary sex characters during the first spawning season. Triploidy is increasingly regarded as an important safety precaution in the generation of transgenic fish (Thorgaard, 1991; Devlin *et al.*, 1994) and in the prevention of the loss of biodiversity resulting from escaped fish in areas of intensive aquaculture (Johnstone, 1995); thus the evaluation of the fertility, however limited, of mature triploid rainbow trout

acquires further relevance. The objectives of Chapter V are (1), the assessment of the reproductive potential of maturing triploid males when crossed to normal (diploid) females, and (2), the characterisation in terms of ploidy and viability of any potential offspring.

There is increasing evidence in the literature on the variation observed in the effects of induced triploidy on male and female teleosts. In general, the gonadal development and endocrine profiles of triploid males appear similar to diploid males (Lincoln, 1981; Benfey *et al.*, 1986, 1989; Kitamura *et al.*, 1991; Sumpter *et al.*, 1991; Kobayashi *et al.*, 1993; Nakamura *et al.*, 1993) while triploid females develop string-like ovaries and show very low steroid hormone levels (Benfey and Sutterlin, 1984; Lincoln and Scott, 1984; Nakamura *et al.*, 1987; Sumpter *et al.*, 1991; Cerisola and Dazarola, 1996).

Only in recent years the development of innovative cytogenetic techniques have facilitated the study of chromosomal interaction and pairing during meiosis in teleosts (Foresti *et al.*, 1983; Wise and Nail, 1987; Lin and Yu, 1991; Oliveira *et al.*, 1995). The combination of these techniques with the generation of monosex populations and the manipulation of phenotypic sex by hormonal administration allows for the investigation of sex-related differences during the process of chromosome synapsis in teleosts (Tayamen and Shelton, 1978; Okada, 1985; Mair *et al.*, 1987; Johnstone, 1995). This is particularly feasible in the Nile tilapia ($n=44$, Jalabert *et al.*, 1971; Arai and Koike, 1980; Nijhar *et al.*, 1983; Majumdar and McAndrew, 1986), a species offering the advantages of a well-documented reproductive, genetic and karyological background, early puberty, an XX-XY system of sex determination and well-established protocols for triploidy induction

and sex-reversal (Hussain *et al.*, 1991; Mair *et al.*, 1991; Foresti *et al.*, 1993; Mair and Santiago, 1994; Vera-Cruz and Mair, 1994). Using the Nile tilapia as an experimental fish model, Chapter VI was designed to test the hypothesis that sex-specific pairing behaviour during meiotic prophase I may explain the discordance in the degree of meiotic progression observed in male and female triploid teleosts.

Each Chapter contains an Introduction and Materials and Methods section of specific relevance to the subject(s) covered; Discussion sections in the Chapters have been produced to address specific questions of interest to the Chapter. Chapter VII consists of a General Discussion of the four experimental chapters and summarises the Conclusions. Overall aims of the present Thesis are:

- (a), the study of the feasibility of triploidy induction in two commercially important species representative of cold and warm-water teleosts (rainbow trout and Nile tilapia), using two different techniques for ploidy manipulation (heat and pressure shocks);
- (b), to obtain an overall assessment of the performance of triploid teleosts under controlled culture conditions, with particular emphasis on the effects of triploidy induction on the reproductive characteristics of these animals; and
- (c), to investigate the causes resulting in the differential effects of triploidy induction on the gonadal development of male and female teleosts.

CHAPTER *II*

GENERAL MATERIALS AND METHODS

Materials and methods sections describing specific protocols of particular relevance for individual chapters are included within Chapters III to VI. Only the description of the materials and methods of general interest used throughout the studies for fish maintenance and handling, preparation of the diets for sex reversal, ploidy determination and histological studies are included in this Chapter.

2.1 *Fish origin and maintenance*

2.1.1 *Studies conducted at the University of California, Davis*

Studies on morphometric parameters and fertilising ability of triploid rainbow trout (Chapters III and V) were conducted at the facilities of the Institute of Ecology of the University of California at Davis, U.S.A. Fish used in these experiments were from two local strains (RTJ, 'Javier' and 'RTD', Davis) obtained from Mount Lassen Hatchery, Red Bluff, California. All the fish used for the experiments conducted at Davis (39°N 122°W) were maintained under natural photoperiod regimes and ambient temperature (T= 10-18 °C) in aerated standard fibreglass circular tanks under flow-through systems. Fish were fed twice daily with a commercially-available dry trout diet (Silver Cup, Corvallis, Oregon, U.S.A.) at rations as specified in the company's feed tables.

2.1.2 *Studies conducted at the University of Stirling, Scotland*

Studies on the gametogenesis of triploid rainbow trout and meiosis in diploid and autotriploid Nile tilapia were conducted at the facilities of the Genetics and Reproduction Research Unit of the Institute of Aquaculture, University of Stirling, Scotland. Nile tilapia used in Chapter VI were obtained from broodstock held at the Tropical Aquarium of the Institute of Aquaculture, and originate from a Lake Manzala (Egypt) population. These broodfish were kept in aerated 100 L rectangular glass tanks linked to a recirculating system. The water temperature was maintained at 27 ± 2 °C with a 12L:12D photoregime. Biological filters in the recirculation system were cleaned twice a month and the fish were fed daily on a commercial cal trout feed (Trouw Ltd., Northwich, U.K. and/or Ewos Ltd., Bathgate, Scotland) n° 3 to 5 (54-40% protein).

2.2 *Fish handling*

Animal monitoring and handling in all experiments described in this study conformed to the regulated procedures on living animals established by the United Kingdom Animals (Scientific Procedures) Act of 1986. Handling stress during blood collection for ploidy determination, egg stripping or sperm collection was minimised by anaesthetising the fish in methyltricainesulfonate (MS-222, Sigma Chemical Company, St. Louis, Missouri, U.S.A.) or in ethyl-p-aminobenzoate (benzocaine; Sigma Chemicals, Poole, England) at a 1:10,000 dilution in water. After completing the various handling tasks, the fish were transferred into clean aerated fresh water where they generally recovered within 3-5 minutes.

2.3 Preparation of the diets for sex reversal

Diets used for sex reversal of tilapia female genotypes in Chapter VI were supplemented with 50 mg/kg of 17 α -methyltestosterone (Sigma-Aldrich, Poole, U.K.). Strict precautions were observed when handling the steroid, wearing a protective laboratory coat, close-fitting mask, surgical gloves and performing all manipulations in the fume cupboard.

For the treatment of the diets, a 10 mg/ml stock solution of the steroid was prepared, using reagent-grade 100% ethanol (BDH, Dorset, U.K.) as a solvent. N° 3 feed pellets (Trouw Ltd., Northwich, U.K.) were ground using an electric coffee grinder, sieved to 500 -1,000 μ m particle size, weighed and spread into aluminium foil-covered plastic trays forming a layer approximately 5 mm deep. To achieve the desired steroid concentration, 0.5 ml of the steroid stock solution were added to 9.5 ml of 100% ethanol, and the solution transferred into a small hand plant sprayer. The steroid solution was evenly spread onto the food from close (10-15 cm) range to prevent loss of solution, while continuously turning the food with a plastic spatula in order to ensure proper mixing.

Once the steroid solution was finished, 3 ml of 100% ethanol were added into the sprayer and sprayed onto the food to ensure that no traces of the steroid remained in the bottom of the sprayer. Untreated (control) food was ground, sieved and sprayed with 100% ethanol in a similar manner. The food was then thoroughly mixed, left to dry overnight and stored in airtight containers at -20 °C until use.

2.4 Ploidy determination

2.4.1 Sample collection

Ploidy was determined by measuring the nuclear size (in the experiments described in Chapters III, IV and V) or the whole cell size (in the experiments described in Chapter VI) of red blood cells with a Coulter Counter model ZM connected to a C-1000 Channeliser (Coulter Electronics Inc., Hialeah, Florida, U.S.A., Figure 2.1, A). The protocol used follows those of Johnson *et al.* (1984) and Wattendorf (1986), with several minor modifications. After anaesthesia, blood samples were taken from the caudal vein by using either 23-gauge or 25-gauge sterile hypodermic needles fitted into sterilised 1 ml syringes (Terumo Europe N.V., Leuven, Belgium) treated with sodium heparin (1000 USP units/ml) or Alseyer's anticoagulant (2% glucose, 0.8% tri-sodium citrate and 0.4% sodium chloride in distilled water). Approximately 1 μ L of blood was aspirated from the syringe with an Eppendorf pipette and added to an Acuvette sample vial (Coulter Electronics Inc., Hialeah, Florida, U.S.A.) filled with 10 ml of Isoton II electrolyte solution (Appendix 1) and carefully mixed by repeatedly inverting the vial. For the determination of nuclear red blood cell size, 2 μ L of the lysing agent Zapoglobin[®] (Curtin Matheson Scientific, Boston, Massachusetts, U.S.A.) were added to the vial prior to blood addition. The vials were stored at 4°C until Coulter Counter analysis.

2.4.2 Sample analysis

The Coulter Counter model ZM was set up with a 70 μ m aperture tube. Once precalibration was completed by the half-count method described in the

owner's manual, optimum settings for the Counter and Channeliser were determined.

The following settings were used: current, 100; full scale, 10 mA; polarity, negative; lower threshold, 10.0; upper threshold, 99.9; attenuation, 2 and preset gain, 1. The Coulter Channeliser was used with the following settings: base channel threshold, 5.0 and window width, 50.0. Before samples were analysed a background count was determined on the electrolyte (and lysing agent solution when applicable); with the manometer select switch at 100 μ L, a count of less than 250 particles was considered acceptable.

Vials containing whole or lysed blood cells were placed on the sample stand and raised into place so that the aperture tube of the Counter was immersed in the solution. The orifice stopcock was then opened and when particles began passing through the aperture tube, as indicated by vertical pulses on the ZM monitor, the orifice monitor was checked to assure that no blockage had occurred. Particle counts were performed on 1,000 cells or nuclei in the peak channel and two replicate analysis were run per sample. Data output were analysed using the Multisizer AccuComp[®] Analysis software (Coulter Electronics Inc., Hialeah, Florida, U.S.A., Figure 2.1, B-E).

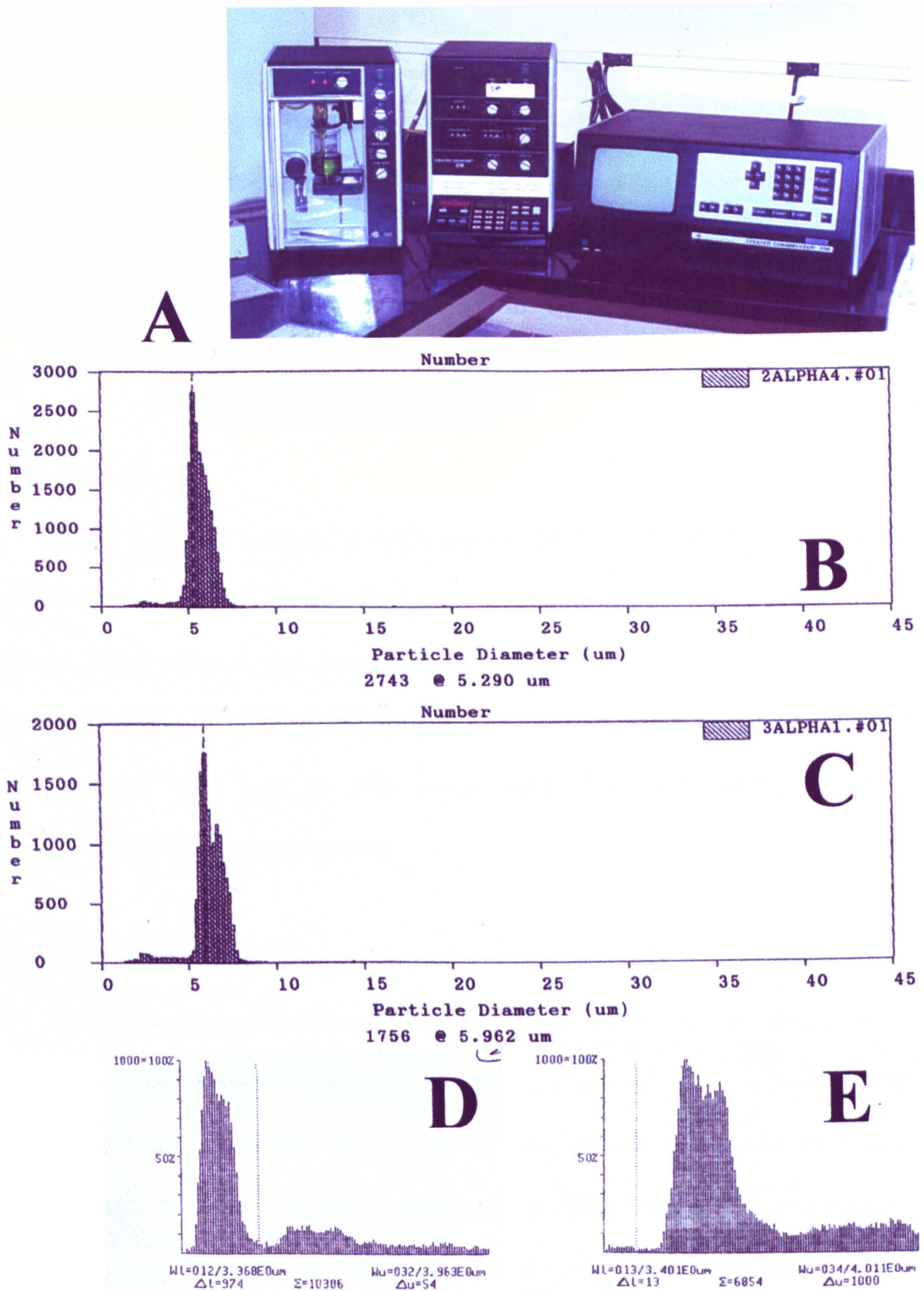


Figure 2.1. A, Coulter Counter (left) and Channeliser (right) used for ploidy determination. B, representative whole-erythrocyte analysis histogram from a diploid individual (tilapia). C, analysis histogram from a triploid individual (tilapia). D, representative nuclear erythrocyte analysis histogram from a diploid individual (trout). E, analysis histogram from a triploid individual (trout).

2.5 *Histology protocols*

Tissue fixation, processing, embedding, sectioning and staining protocols for the experiments described in Chapters IV and V were carried out according to the routine procedures used at the Histopathology Laboratory of the Institute of Aquaculture, University of Stirling. These protocols generally follow those described by Carleton (1980).

2.5.1 *Tissue fixation*

Freshly collected gonadal samples were fixed in a 10% phosphate buffered formaldehyde solution (Appendix 2). To ensure proper fixation, a ratio 1:10 tissue fragment: fixing solution was consistently maintained in the fixing vials and the fixative was replaced by fresh fixing solution after the initial 24 hr of fixation. Tissue samples were kept in 10% phosphate-buffered formaldehyde for a minimum of 72 hr prior to processing.

2.5.2 *Tissue processing, embedding and sectioning*

Fixed fragments of the gonads were cassetted, labelled and processed in an automated tissue processor (Shandon Scientific, Cheshire, U.K.) following the processing schedule described in Appendix 3. After paraffin infiltration the tissue fragments were blocked in suitably sized moulds using molten wax and rapidly cooled on a cold plate. Tissue blocks were trimmed to bring the tissue to the surface of the block and whenever necessary (i.e. gonadal fragments containing large oocytes) surface decalcification was carried out by treating the surface of the block with a rapid decalcifier for 30-60 min (RDC-Histolab, London, England). The blocks were then washed in tap water, cooled on a cold plate and sections

(thickness= 5-6 μm) were cut on a motorised retracting microtome (Reichert-Jung 2050, Nussloch, Germany) using standard disposable microtome blades. Thin sections were floated on a distilled water bath at 40 °C and collected on glass slides (Superfrost[®] BDH, Dorset, U.K.). Tissue sections on the glass slides were labelled, cured in a warm plate at 70 °C for 30-50 min and dried overnight in an oven at 60 °C before staining.

2.5.3 Staining procedures

For general observations all sections were stained with the haematoxylin and eosin stain, following the protocol specified in Appendix 4. The periodic acid-Schiff (PAS) reaction, Heidenhain's iron haematoxylin and Masson's trichrome were used in replicate sections of the gonads for the histological analysis of gametogenesis described in Chapter IV. The PAS reaction was used because of its compatibility with formalin fixation and its efficiency to demonstrate basement membranes, lipochrome pigments and polysaccharides. Heidenhain's iron haematoxylin was used as a counterstain because of its affinity for nuclear components such as chromatin and nucleoli (Carleton, 1980). Masson's trichrome was used on the basis of its ability to differentiate the granulosa and theca layers of developing oocytes. Specific protocols for these stains are provided in Appendices 5 and 6. Stained sections were mounted on synthetic mounting medium (Pertex[®], Histolab, London, England) and stored in opaque dust-free histological boxes until examination.

2.6 Classification of cell stages in the gametogenesis of rainbow trout

The classification of gametogenesis stages described in Chapter IV and the assessment of gonadal maturation in triploid rainbow trout males described in Chapter V are based in previous studies on the gametogenesis of this species. Spermatogenic stages were identified following the criteria of Billard (1983, 1986, 1992). The classification of gonadal cell stages in females primarily follows that of Bromage and Cumaranatunga (1988). Additional references consulted on the morphology of gonadal cell stages include those by Van der Hurk and Peute (1979), Takashima *et al.* (1980), Van der Hurk and Slof (1981), Lebrun *et al.* (1982), Van der Hurk *et al.* (1978, 1982), Browder (1985), Guraya (1986), Kinne (1991), and Tyler and Sumpter (1996). Detailed morphological descriptions of the cellular classes observed during the gametogenesis of the rainbow trout are provided in Sections 1.7.3 and 1.7.6 of this Thesis.

CHAPTER *III*

MORPHOMETRIC PARAMETERS IN TRIPLOID RAINBOW TROUT,

ONCORHYNCHUS MYKISS

3.1 Summary

Triploid rainbow trout were produced by a 13-minute, 27°C heat shock applied to pooled eggs collected from three mature females 11 minutes post-fertilisation. Treated (triploid) and control (diploid) siblings were raised separately at similar density, feeding and water quality conditions, and several morphometric parameters (fork length and the weight of the body, eviscerated carcass, gonads, pituitary and liver) were recorded at five sampling times over a 44-month period.

Ploidy determination by measurement of nuclear size at 5 months post-hatching revealed that 46% of the individuals analysed in the treated group were triploid. No significant differences in sex ratios, body weight or condition factors were consistently observed between the triploid and diploid groups throughout the experimental period; however the weight of the eviscerated carcass was on average 20% higher ($P < 0.05$) in triploid fish compared to diploid fish at 20 and 44 months post-hatching.

Triploidy induction resulted in the suppression of sexual maturation in females. Gonadal weight was in general similar between triploid and control males, whereas triploid females had very low gonadal weight and gonado-somatic indices (maximum gonado-somatic index = 0.03% at 44 months of age). The commercial relevance of these results and the possible applications of triploidy induction for the production of large (over 1.5 kg of body weight) rainbow trout and/or the reproductive containment of farmed stocks are discussed.

3.2 Introduction

Cultivated polyploid species today play an important part in the supply of food, as polyploids exhibit, in general, good growth performance and improved production yields. The manipulation of ploidy through the physical treatment of fish gametes has equally become an important commercial tool in the aquaculture industry, in particular the production of triploid individuals (Swarup, 1959; Chourrout, 1980; Thorgaard *et al.*, 1981).

The main goal pursued when producing triploid fish is their potential sterility, which is assumed to arise from the triploid genome of the germ cells resulting in aberrant meiosis and leading to the suppression or substantial alteration of gametogenesis (Thorgaard and Gall, 1979). The idea of sterilising part or all of the harvestable portion of a stock is attractive as a method to reduce the deleterious effects (i.e. growth retardation, deterioration of flesh quality and increased mortality) characteristic of sexual maturation in many teleosts, particularly in the salmonids, with the added advantage of a possible diversion of metabolisable energy from gonadal to somatic growth (Utter *et al.*, 1982; Thorgaard, 1986; Ihssen *et al.*, 1990; Hussain, 1996).

By not having to harvest the fish before the time of sexual maturation, triploidy allows for the distribution of sales throughout the year, resulting in more flexible management of the stocks. In addition, if only sterile individuals are marketed, the producer remains in control of the line(s), capitalising on the profit from frequently expensive and time-consuming selection programmes (Thorgaard, 1983). Sterility also facilitates the release of exotic species into new ecosystems, prevents the loss of biodiversity caused by escaped fish in areas of

intensive farming, and is regarded as an important safety precaution for the testing of genetically manipulated (i.e. transgenic) fish (Thorgaard, 1991; Devlin *et al.*, 1994; Johnstone, 1995).

Triploidy also allows an additional dimension to the generation of hybrids, with the potential for increased viability in triploid hybrids due to the presence of a supplementary maternal genome. In some specific cases (e.g. disease resistance), the relative superiority of triploid hybrids may be of primary importance. An example is the hybrid between the rainbow trout and the brook trout, *Salvelinus fontinalis*. These hybrids maintain the favourable commercial traits of the rainbow trout while inheriting from the paternal *S. fontinalis* genome the resistance to viral haemorrhagic septicaemia (VHS), a disease to which the rainbow trout is very sensitive (Scheerer and Thorgaard, 1983). Triploidy is thus now regarded as a route to increase the number of viable hybridisations (Scheerer and Thorgaard, 1987; Sutterlin *et al.*, 1987; La Patra *et al.*, 1993; Habicht *et al.*, 1994).

The refinement of the techniques developed for the manipulation of ploidy in fish has been paralleled by an increased interest in the viability and performance of triploid individuals. The effects of artificially induced triploidy on the metabolism (Oliva-Teles and Kaushik, 1987, 1990), physiology (Happe *et al.*, 1987; Virtanen *et al.*, 1990; Biron and Benfey, 1994; McCarthy *et al.*, 1996; Stillwell and Benfey, 1996), cytological parameters (Small and Benfey, 1987; Konishi *et al.*, 1991), tissue and organ structure (Aliah *et al.*, 1990; Krueger and Kohlmann, 1993; Greenlee *et al.*, 1995), behaviour (Kitamura *et al.*, 1991; Kavumparath and Pandian, 1992) and flesh quality and preservation potential (Ehira and Maruoka, 1991) on several species of teleosts have been investigated.

Commercially relevant traits such as growth and survival have been relatively well documented in triploid salmonids. However, information on these parameters remains inconclusive, as some studies have indicated a relative superiority of triploids compared to diploid forms (Habicht *et al.*, 1994), some suggest inferior performance of triploids (Ihssen *et al.*, 1991; Simon *et al.*, 1993; Galbreath and Thorgaard, 1995) while others report no significant differences between both forms (Myers and Hershberger, 1991; Carter *et al.*, 1994). These conflicting results may reflect variations in culture conditions and/or genetic differences among the strains studied (Guo *et al.*, 1990; Myers, 1991). In addition, most of the research to date has focused on the development of juvenile triploid fish covering the first sexual maturation, while information concerning the performance of later stages remains scarce (Thorgaard, 1986).

The objective of this Chapter is to provide a long-term evaluation of commercially-relevant morphometric parameters in triploid rainbow trout, in comparison to diploid forms, when maintained under controlled culture conditions.

3.3 *Materials and Methods*

3.3.1 *Experimental animals*

Triploid rainbow trout were produced from broodstock selected from crosses of RTJ ('Javier') and RTD ('Davis') Mt. Lassen-derived strains (2 year-old, 1.2-1.5 kg body weight). An approximate total number of 2,000 eggs were stripped from three mature females, pooled and fertilised with pooled sperm collected from four different males (water temperature = 13.6 °C). Triploidy was induced in half of the fertilised eggs by a 13-minute, 27 °C heat shock applied 11 minutes after fertilisation. Treated and untreated eggs were incubated separately in standard wire-mesh egg baskets suspended in hatchery troughs at 13 ± 2 °C. Unfertilised or dead eggs were removed daily by the siphon egg picking method (Leitritz and Lewis, 1980). 46 day post-fertilisation-hatchlings were transferred to 4 L rearing pots where they were kept for 40 days. 3 month-old alevins were moved to circular fibreglass tanks 1.2m in diameter. Treated and untreated siblings were raised in separate tanks at similar density, feeding and water quality regimes (T= 10-18 °C, ambient photoperiod).

3.3.2 *Ploidy determination*

Ploidy was determined in 5 month-old juveniles by measuring the nuclear size of erythrocytes with a Coulter Counter and Channeliser (Coulter Instruments, Colorado, USA)(Wattendorf, 1986). 'True' triploid and diploid (control) siblings were transferred after ploidy determination to separate fibreglass tanks 2.5 m in diameter. 1.5 yr old-adults were transferred to 5 m flow-through circular tanks where they were kept until the end of the sampling period.

3.3.3 Measurement of morphometric parameters

Triploid and diploid groups were sampled at 7, 15, 20, 25 and 44 months post-hatching by overanaesthetising randomly selected individuals in a 0.01% solution of methyltricainesulfonate (MS-222, Sigma Chemical Co., St. Louis, Missouri, USA). After ploidy reassessment by Coulter Counter, the following parameters were recorded for each individual sampled:

- (a), Fork length (the length of the fish measured from the tip of the snout to the point of division of the caudal fin, expressed in centimetres);
- (b), Body weight (expressed in grams);
- (c), Carcass weight (weight of the fish after dissection of the abdominal contents, including the gastrointestinal tract, liver and digestive glands, abdominal fat and gonads, expressed in grams);
- (d), Gonadal weight (including gonadal ducts, expressed in grams);
- (e), Pituitary weight (expressed in grams);
- (f), Liver weight (after dissection of the gall bladder, expressed in grams).

In addition, the following indices were computed for each individual sampled:

$$(a), \text{Condition factor, } K = \frac{\text{body weight}}{(\text{fork length})^3} \times 100;$$

$$(b), \text{Condition factor based on carcass weight, } K_c = \frac{\text{carcass weight}}{(\text{fork length})^3} \times 100;$$

$$(c), \text{Gonado-somatic index, } GSI = \frac{\text{gonadal weight}}{\text{body weight}} \times 100;$$

$$(d), \text{Hepato-somatic index, } HSI = \frac{\text{liver weight}}{\text{body weight}} \times 100.$$

Sex was ascertained in every sampled individual by histological examination of paraffin-embedded gonadal sections (see Chapter IV).

3.3.4 *Statistical Analysis*

Morphometric parameters were compared between ploidy groups by the Student's *t*-test. Differences in condition factors, hepato-somatic and gonado-somatic indices were investigated by Student's *t*-test on arcsine-transformed values. Sex ratios were tested for goodness-of-fit to a 1:1 ratio in each ploidy group by using the adjusted *Chi-square* test. Differences were considered significant at the $P < 0.05$ level (Zar, 1996).

3.4 Results

3.4.1 Ploidy determination

136 juveniles were analysed for nuclear red blood cell size in the untreated group. 135 (99.26%) were diploid. The mean nuclear diameter value for the diploid juveniles was $3.66 \pm 0.02 \mu\text{m}$ (average \pm s.e.m.). A total number of 296 juveniles were analysed in the treated group. 135 juveniles were triploid, and 161 were non-triploid fish. The mean nuclear diameter value observed in triploid juveniles was $4.26 \pm 0.03 \mu\text{m}$ (average \pm s.e.m.). Triploid yield was 45.61%.

3.4.2 Sample size and sex ratios

A total number of 160 individuals were examined during the five samples conducted (79 diploid and 81 triploid fish). Mortalities did not significantly differ between the triploid and diploid group during the experimental period. The number and sex of the individuals analysed per sample is presented in Table 3.1. Only one diploid male and one triploid male were available for examination at 44 months post-hatching. Due to difficulties in the dissection of the gland, pituitary weights could only be recorded during the last three samples. Sex ratios in the diploid and the triploid group did not significantly depart from the expected 1:1 ratio.

Months post-hatching	Males		Females	
	Diploid	Triploid	Diploid	Triploid
7	11	9	11	11
15	13	11	8	9
20	4	4	6	6
25	10	10	10	10
44	1	1	5	10
TOTAL	39	35	40	46

Table 3.1. Sample size and observed gonadal sex in diploid and triploid groups at the five sampling stages.

3.4.3 Morphometric parameters

3.4.3.1 Fork length, Body weight and Condition factor (K)

Although mean fork length was significantly lower in the triploid group compared to the diploid group at 7 months post-hatching, this trend was not maintained in subsequent samples (Figure 3.1). On the contrary, triploid males were significantly longer (47.5 ± 2.12 cm, average \pm standard deviation) than diploid males (43.7 ± 0.62 cm) at 20 months post-hatching. No significant differences in body weight were observed between ploidy groups, with the exception of the last sample (44 months post-hatching), when triploid fish were significantly heavier ($5,200 \pm 793$ g, average \pm s.d.) than their diploid counterparts ($4,333 \pm 532$ g). However, sex-related differences in body weight were significant between ploidy groups at 20 months of age (when triploid males outperformed diploid males) and 25 months of age (when triploid females outperformed diploid females, Figure 3.2).

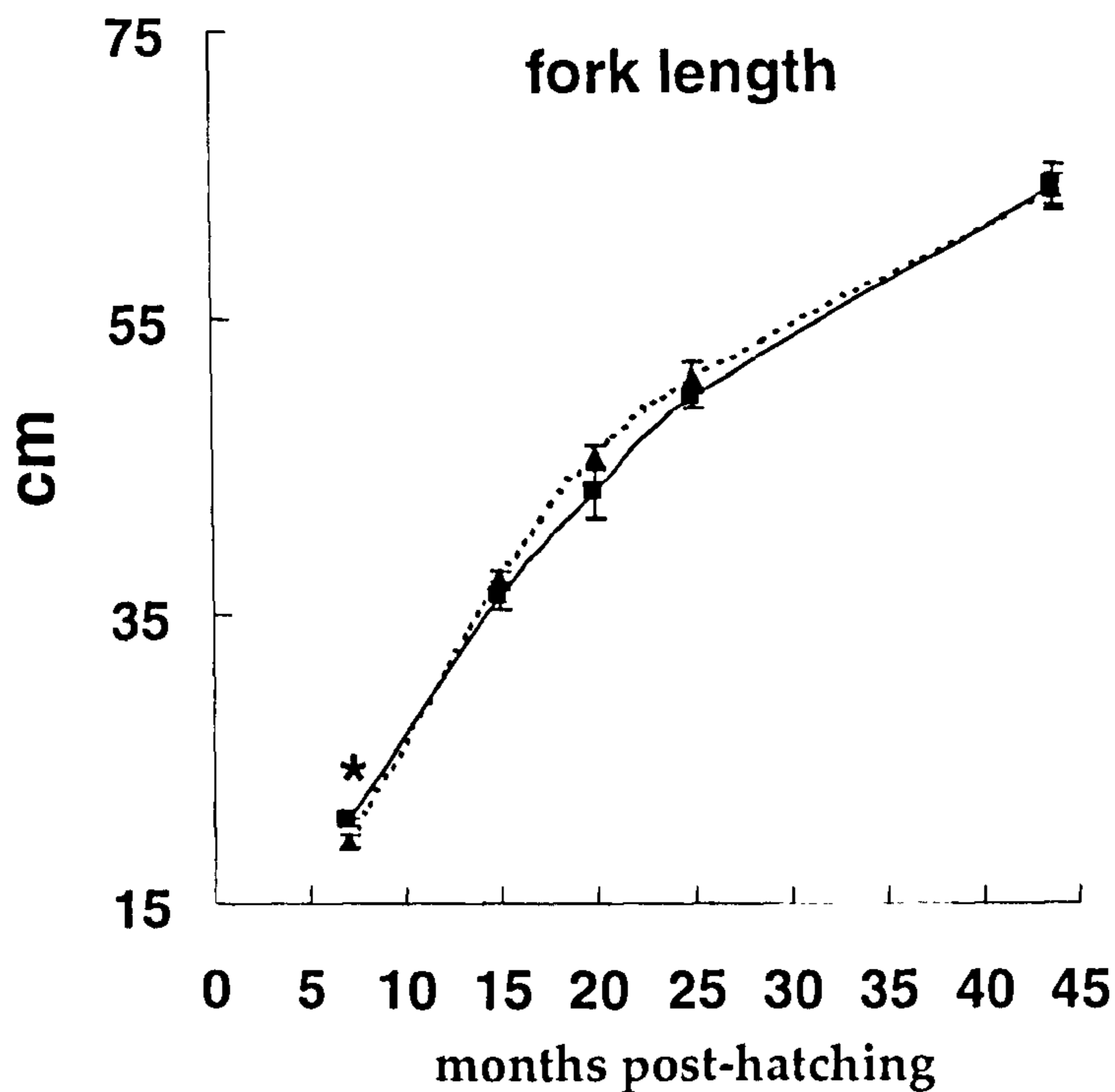


Figure 3.1 Plot of the average fork length in diploid (■) and triploid (▲) fish during the five experimental samples. Values in diploids are joined by a continuous line; values in triploids are joined by a dotted line. Bars represent standard error of the means. The asterisk denotes a significant difference ($P < 0.05$).

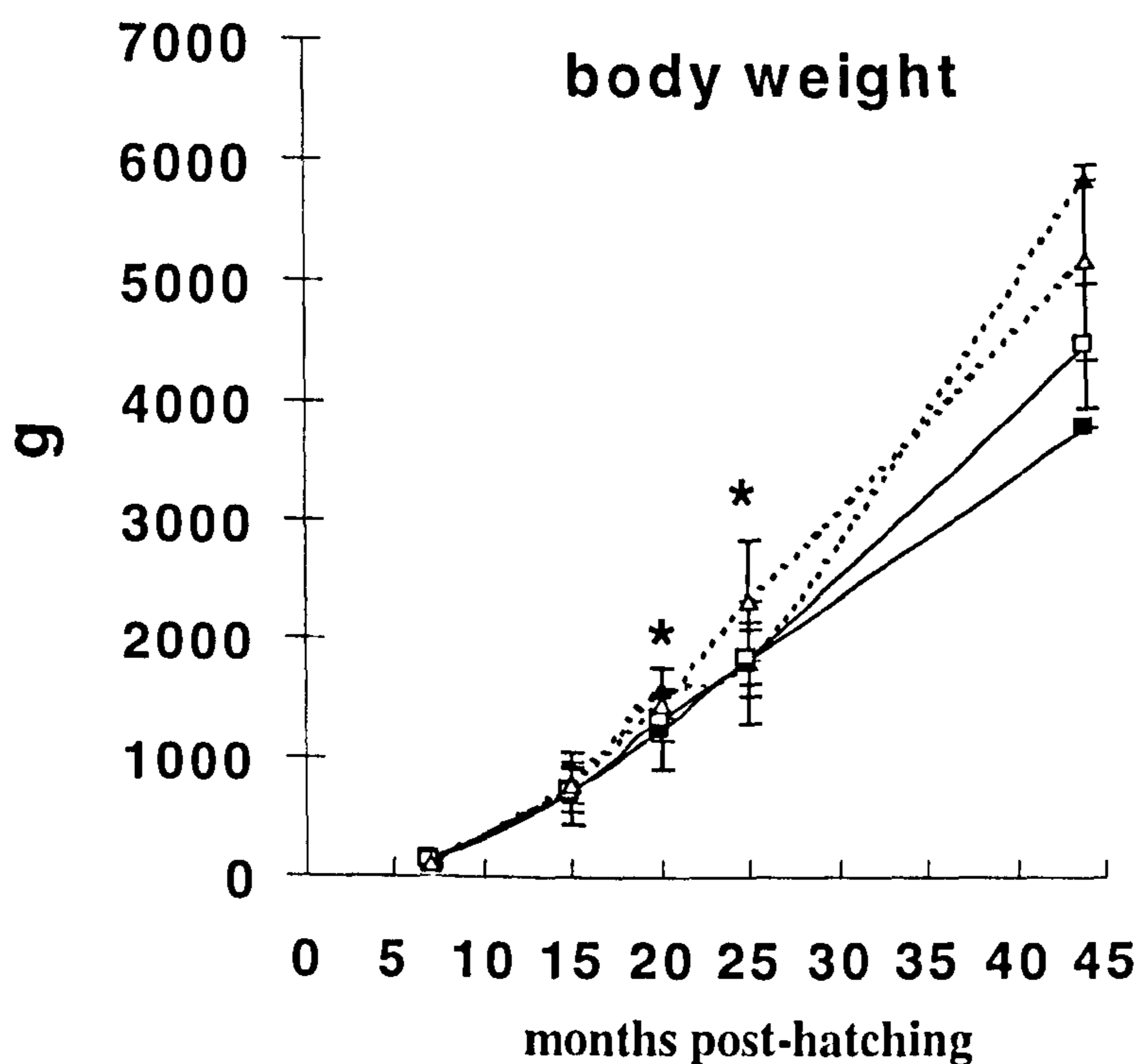


Figure 3.2. Plot of the average body weight in diploid males (■), diploid females (□), triploid males (▲) and triploid females (Δ) for the five samples conducted. Bars represent standard error of the means; asterisks denote significant differences ($P < 0.05$).

Although average condition factors were generally higher in females than in males, no significant differences were observed between ploidy groups throughout the five samples conducted. The only significant differences observed between condition factor averages were sex-related and inverse between sexes, and were noticeable at 25 months post-hatching. Among the females, triploids (average $K= 1.76\%$) significantly outperformed diploid fish (average $K= 1.52\%$), whereas among the males, diploids (average $K= 1.49\%$) outperformed their triploid counterparts (average $K= 1.32\%$) at this sampling time (Figure 3.3).

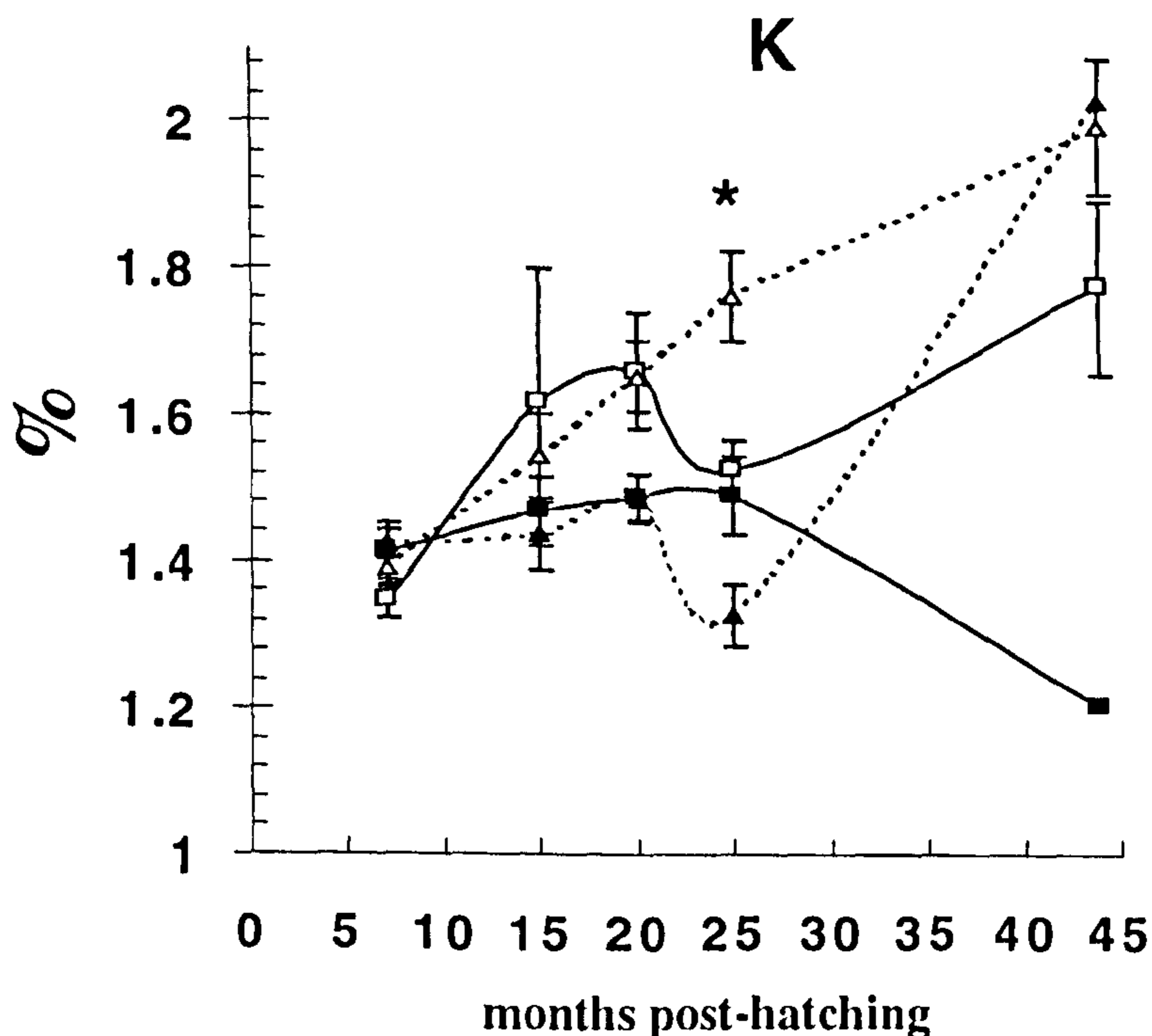


Figure 3.3. Plot of the mean condition factor in diploid males (■), diploid females (□), triploid males (▲) and triploid females (△) for the five samples conducted. Bars represent standard error of the means; the asterisk denotes a significant difference ($P < 0.05$).

3.4.3.2 Carcass weight and Condition factor based on Carcass weight (K_c)

The weight of the eviscerated carcass was significantly higher in triploid fish compared to diploid fish at 20 and 44 months post-hatching (Figure 3.4, left). These differences were paralleled by sex-related differences, since at 20 months of age the weight of the carcass in triploid males ($1,417 \pm 171$ g) was significantly higher than that of diploid males ($1,061 \pm 68$ g), while at 44 months of age triploid females (carcass weight = $4,384 \pm 677$ g) significantly outperformed diploid females (carcass weight = $3,573.6 \pm 439$ g). Differences in condition factor based on carcass weight were only significant between ploidy groups at 44 months post-hatching, and were due to higher K_c values in triploid females ($K_c = 1.69\%$) compared to diploid females ($K_c = 1.42\%$) at this age (Figure 3.4, right).

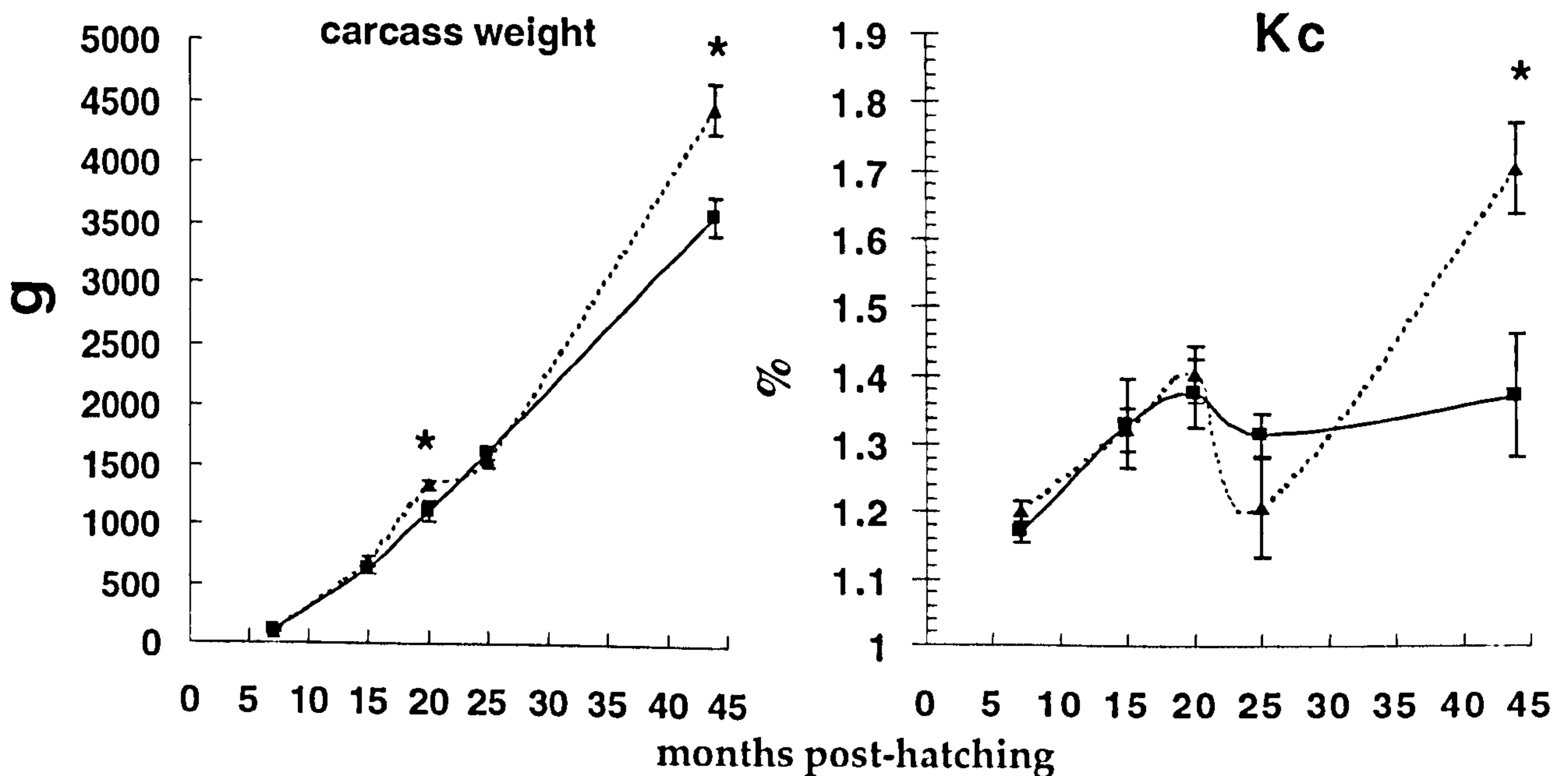


Figure 3.4. Plot of the mean carcass weight (left) and mean condition factor based on carcass weight (right) observed in diploid (■) and triploid (▲) fish during the five experimental samples. Bars represent standard error of the means; asterisks denote significant differences ($P < 0.05$).

3.4.3.3 Gonadal weight, Gonado-somatic index (GSI) and Pituitary weight

Gonadal weight was significantly higher in diploid fish compared to triploid fish from 20 months post-hatching onwards. Differences within sexes were more marked in females than in males. Gonadal weight in triploid females was very low (<15% of the gonadal weight in diploid females) and differed significantly from that of diploid females throughout the entire experimental period (Figure 3.5).

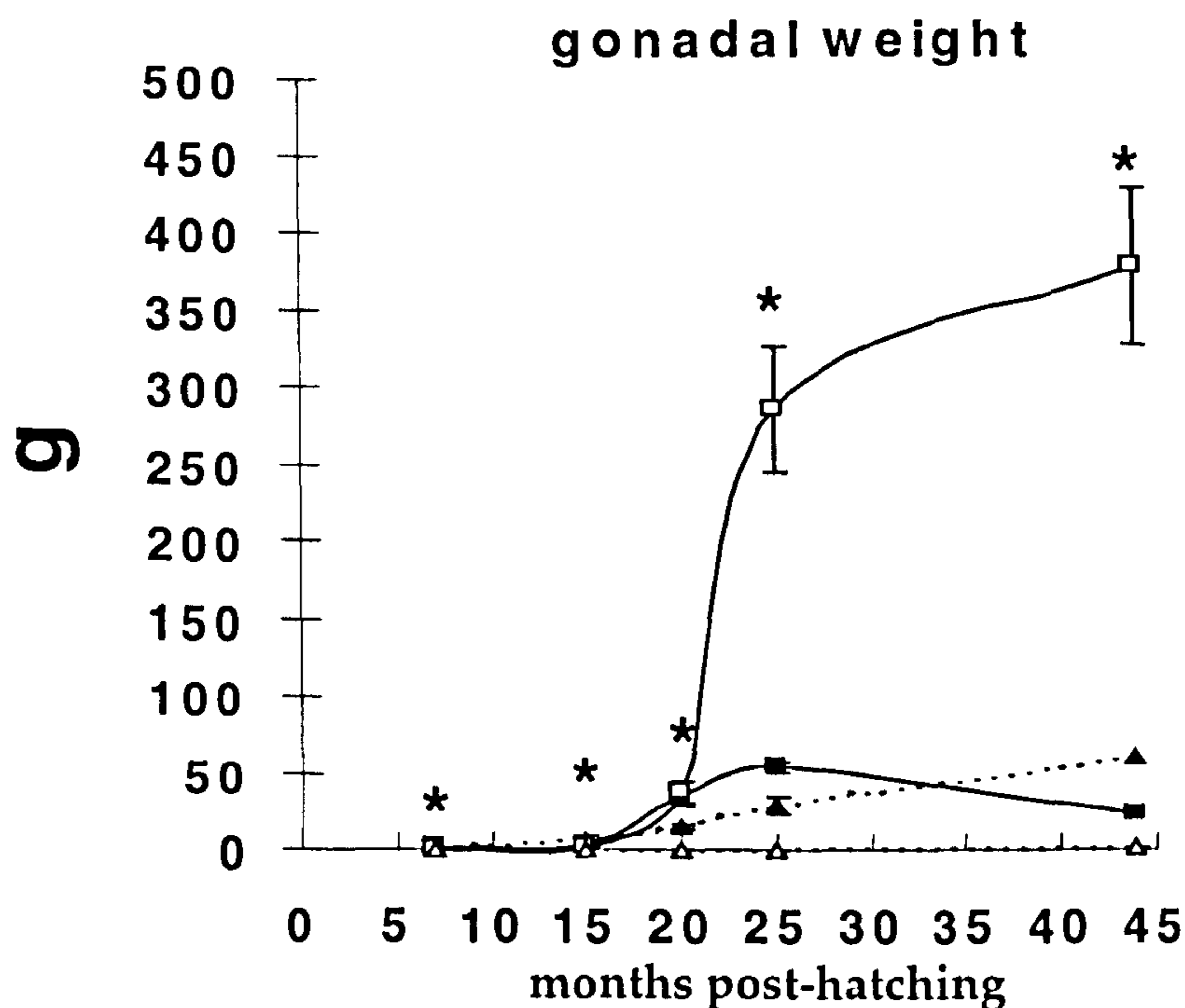


Figure 3.5. Plot of the average gonadal weight observed in diploid males (■), diploid females (□), triploid males (▲) and triploid females (△) during the five samples conducted. Bars represent standard error of the means; asterisks denote significant differences ($P < 0.05$).

Average gonadal weight in triploid males was similar to and in some cases (15 and 44 months post-hatching) even higher (but not significantly so) than that of diploid males, although it was significantly lower at 20 and 25 months post-hatching.

These differences in gonadal weight translated into differences in gonadosomatic indices, which were significantly lower in triploid fish compared to diploid fish at 20, 25 and 44 months post-hatching. GSIs were generally higher in diploid females (reaching a maximum value of 15.1% at 25 months of age), followed by diploid males (with a maximum value of 3%, also at 25 months of age) and triploid males (maximum GSI= 1.5% at 25 months post-hatching), while the lowest GSI values were observed in triploid females (with a maximum value of 0.03% at 44 months post-hatching). Differences in GSIs were more prominent between diploid and triploid females than between diploid and triploid males, with the same pattern of sex-related differences observed in gonadal weights maintained in GSIs (Figure 3.6).

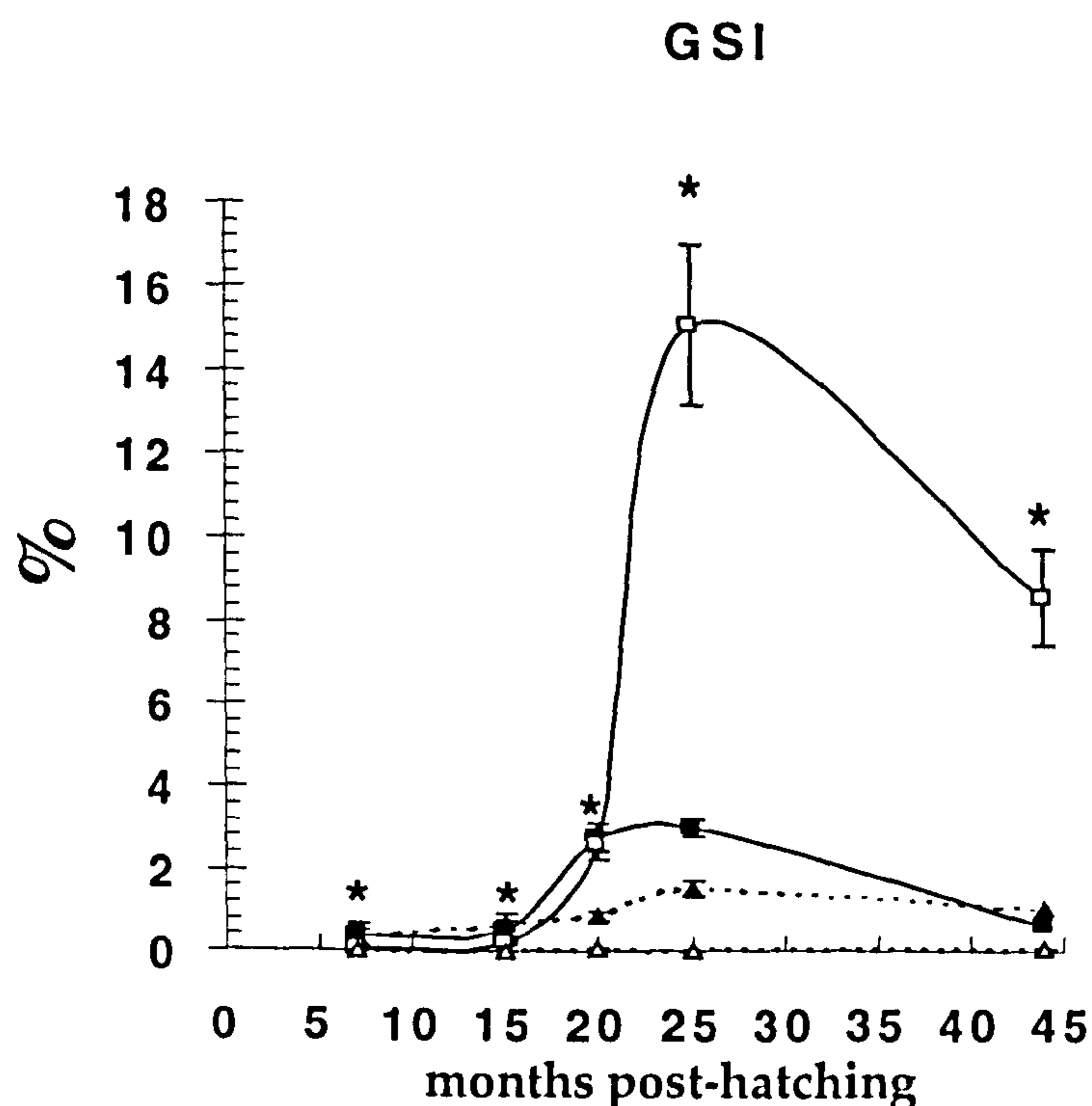


Figure 3.6. Mean gonado-somatic indices observed in diploid males (■), diploid females (□), triploid males (▲) and triploid females (△) during the five experimental samples. Bars represent standard error of the means; asterisks denote significant differences ($P < 0.05$).

The weight of the pituitary gland was significantly higher in triploid fish (mean pituitary weight= 0.016 ± 0.004 g) than in diploid fish (mean pituitary weight= 0.013 ± 0.003 g) at 20 months post-hatching. At 25 months post-hatching, diploid fish had higher pituitary weights (0.058 ± 0.116 g, average \pm standard deviation) than their triploid counterparts (0.017 ± 0.006 g), but these differences were not significant due to the high variation in the values recorded for diploid fish. No significant differences in pituitary weight were observed at 44 months post-hatching between both ploidy groups (Figure 3.7). Equally, no significant differences in pituitary weight were observed within sexes at the three sampling times for which this parameter was available.

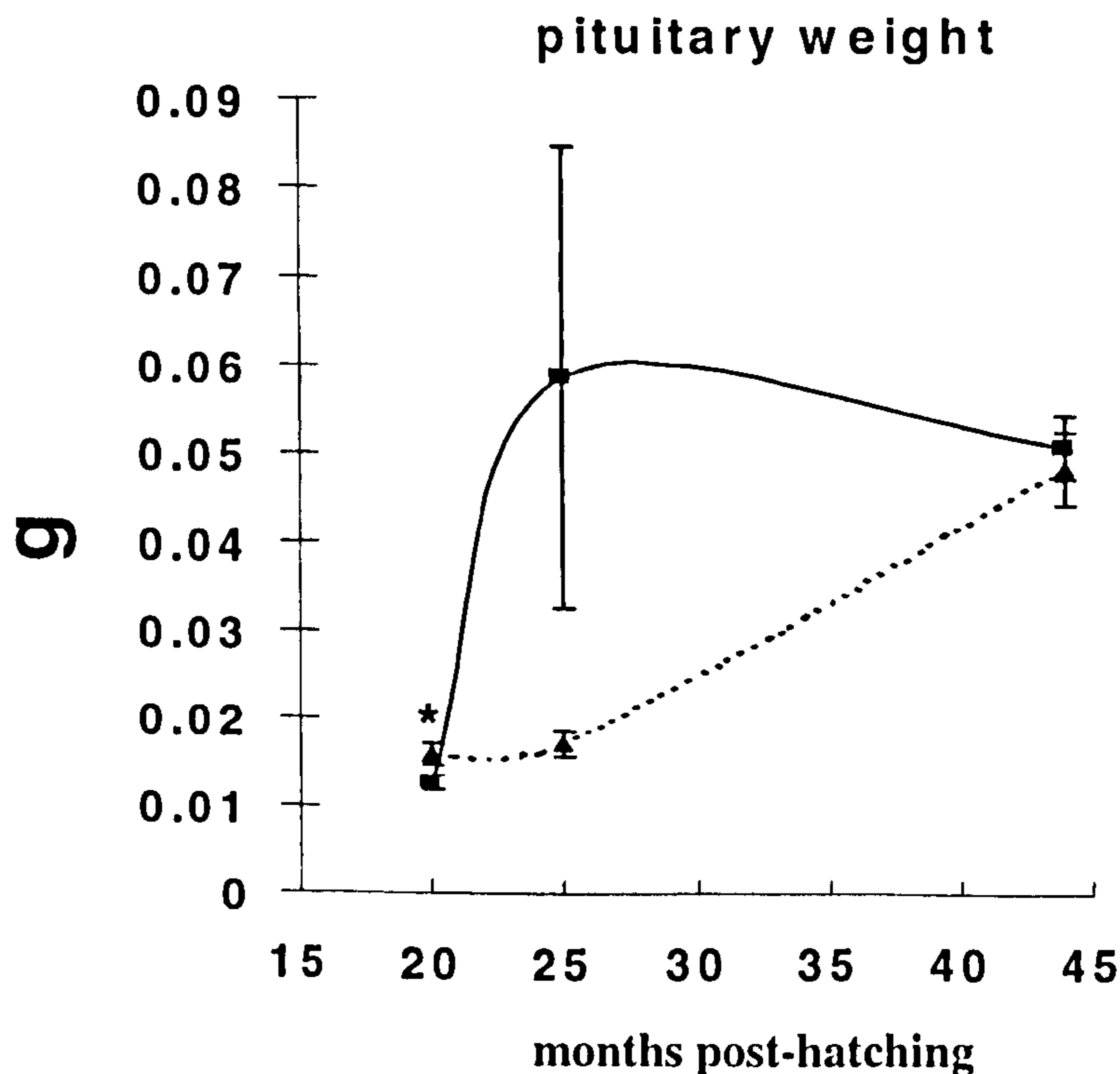


Figure 3.7. Mean pituitary weight in diploid fish (■) and triploid fish (▲) for the five samples conducted. Bars represent standard error of the means; the asterisk denotes a significant difference ($P < 0.05$).

3.4.3.4 Liver weight and Hepato-somatic index (HSI)

The weight of the liver was significantly higher in triploid fish (average liver weight= 24.1 ± 10.4 g) than in diploid fish (mean liver weight= 17.4 ± 3.6 g) at 25 months post-hatching. This difference was attributable to higher liver weights in triploid females compared to diploid females at this age (Figure 3.8), since no significant differences in liver weight were observed between diploid and triploid males at this sampling time, or indeed throughout the experimental period. When expressed as a percentage of body weight, HSIs were also significantly higher in triploid fish (average HSI= $1.12 \pm 0.26\%$) than in diploid fish (average HSI= $0.95 \pm 0.14\%$) at 25 months of age. The general trend, however, was the observation of more variable and higher HSIs in diploid fish compared to triploid fish, manifest as significantly higher hepato-somatic indices in diploids at 15 and 44 months post-hatching (Figure 3.9).

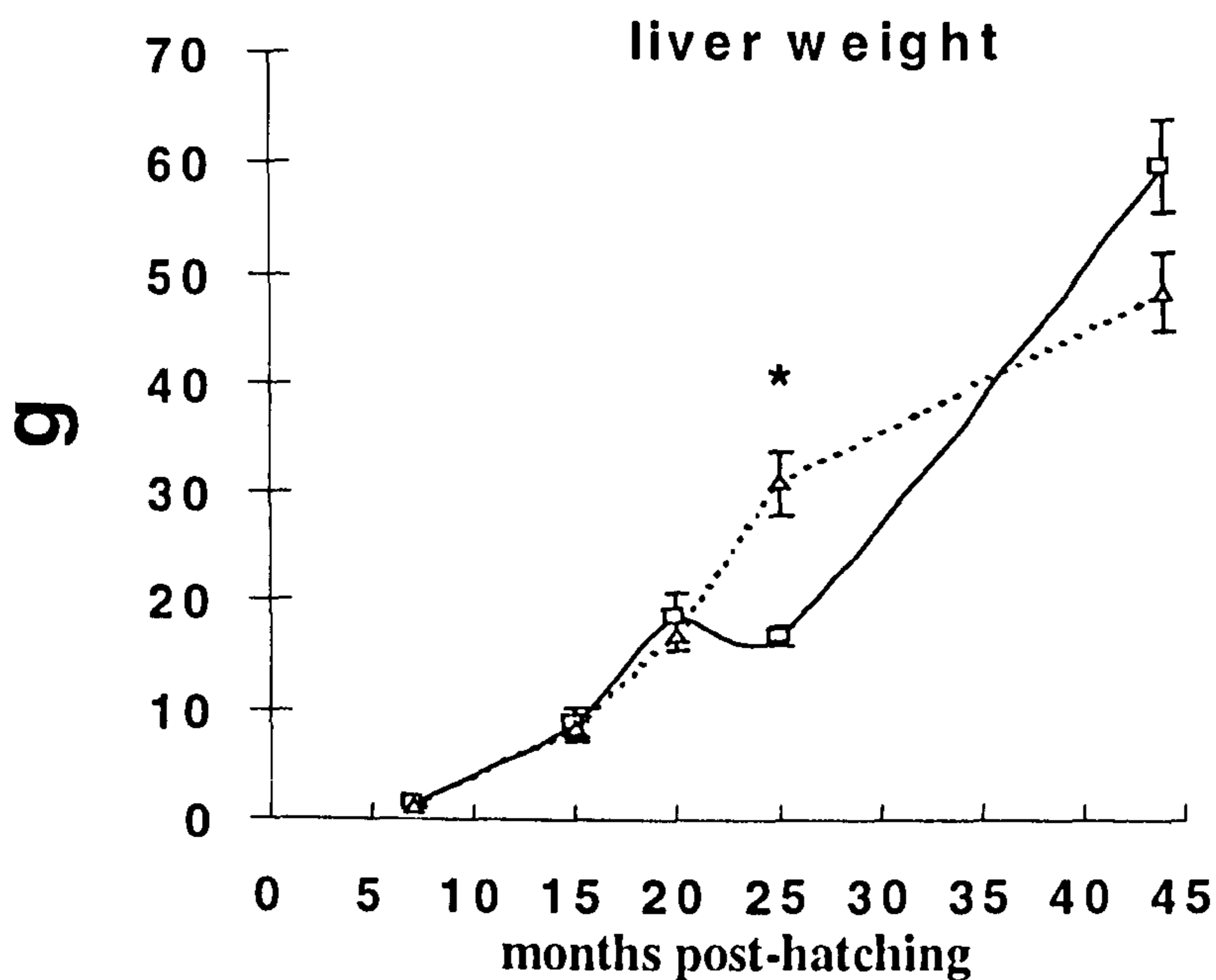


Figure 3.8. Mean liver weights observed in diploid females (\square) and triploid females (Δ) for the five samples conducted. Bars represent standard error of the means; the asterisk denotes a significant difference ($P < 0.05$).

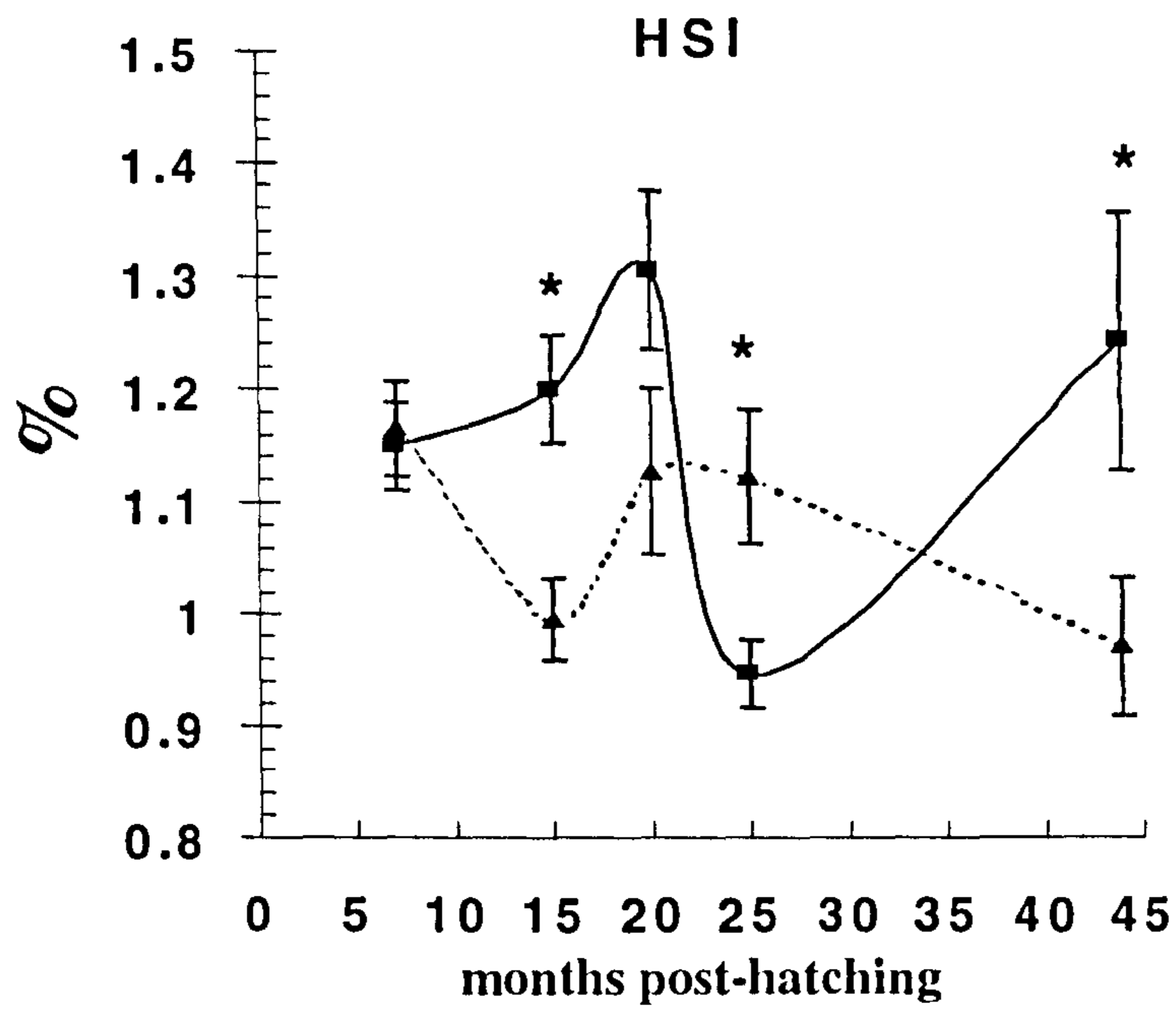


Figure 3.9. Mean hepato-somatic indices observed in diploid (■) and triploid (▲) fish during the five experimental samples. Bars represent standard error of the means; asterisks denote significant differences ($P < 0.05$).

3.5 Discussion

The relatively low triploid yield (46%) observed in this study is in agreement with previous reports on triploidy induction in the rainbow trout suggesting a lower efficiency of heat shocks compared to pressure shocks in the inhibition of the second polar body extrusion (Lou and Purdom, 1984; Lincoln, 1989; Yamazaki and Goodier, 1993). Presumably, higher triploid yields obtained with pressure shocks are related to the shorter duration of the shock and a more uniform treatment of the eggs, although they may also represent variations in the susceptibility of the strains to triploidy induction (Chourrout, 1984; Lou and Purdom, 1984; Benfey *et al.*, 1988; Díaz *et al.*, 1993).

In spite of earlier expectations for high growth in artificially-induced triploid fish, subsequent trials have failed to consistently demonstrate the superiority of triploid stocks for this trait (Ihssen *et al.*, 1991; Myers and Hershberger, 1991; Simon *et al.*, 1993; Carter *et al.*, 1995; Galbreath and Thorgaard, 1995). Data from the present study seem to substantiate the view that triploid fish do not outperform their diploid counterparts, since no significant differences in body weight or condition factors were observed throughout the experimental period. It should be noted, however, that the lack of replications in the present study, conditioned by the facilities available given the time-scale of the experiments conducted, may have resulted in significant tank effects and thus positively or negatively have affected the performance of the triploid group over the controls. Nevertheless, the significantly higher carcass weights observed in triploid fish at 20 and 44 months of age (with the triploid stock outperforming the diploid stock in 220 g and 880 g, respectively), constitute a valuable observation

from a commercial perspective, since the weight of the eviscerated carcass is a better yield indicator than the whole body weight for the processed fish supplier. Triploid rainbow trout thus seem to be desirable for production schemes marketing large, older fish (over 1.5 kg of body weight and/or over 20 months of age), where the gain in carcass weight (19.4% and 24.7% higher in triploid fish at 20 and 44 months of age, respectively) can be fully exploited. Triploidy induction would not provide a clear growth performance advantage in production systems marketing small trout, prior to sexual maturation.

The main impact of triploidy observed in this work was the substantial alteration in the effects of the sexual maturation in rainbow trout, particularly in females. Differences between ploidy groups were generally more evident immediately before and after the first spawning (20 and 25 months post-hatching, see Chapter IV, sections 4.4.2.3 and 4.4.2.4), with a general pattern of triploid females showing almost negligible gonadal growth and very low gonado-somatic indices, and triploid males showing similar gonadal parameters to diploid males. Similarly, the values recorded for the weight of the pituitary and the liver in the triploid group seem to correspond with the alteration in the reproductive physiology caused by triploidy induction. Higher liver weights in triploid females after spawning, for instance, are probably explained by the involvement of this organ in vitellogenesis and nutrient deposition in the ovary of diploid females (Lincoln and Scott, 1984). The generally lower pituitary weight values recorded in triploid fish may well reflect the alteration of the interactions in the hypothalamic-pituitary-gonadal axis resulting from the triploidy induction, although it has been recently shown that the pituitary in triploid rainbow trout remains sensitive to exogenous steroid implantation (Breton and Sambroni, 1996).

The dramatic reduction in gonadal growth observed in triploid females was accompanied by significantly higher values than diploid females in body weight, condition factor, liver weight and hepato-somatic indices at 25 months of age.

There was also a general trend for lower variation in these indices in triploid females than in their diploid siblings throughout the period studied, clearly reflecting the suppression of the effects that sexual maturation and spawning bring about in diploid females. On the contrary, the effects of triploidy induction on rainbow trout males in this study were much less severe and resulted in poor performance, as shown by the similar gonadal weight and gonado-somatic indices observed in triploid and diploid siblings throughout the five samples conducted, and the significantly lower condition factors recorded in triploid males at 25 months of age. These results provide a strong justification for the combination of triploidy induction with sex-control strategies aimed to the generation of all-female populations in the rainbow trout.

CHAPTER *IV*

GAMETOGENESIS IN TRIPLOID RAINBOW TROUT,

ONCORHYNCHUS MYKISS

4.1 Summary

A long-term, quantitative analysis was conducted on the gametogenesis of autotriploid rainbow trout (*Oncorhynchus mykiss*) in order to quantify their degree of germ line development and reproductive potential. Triploid and diploid (control) trout siblings were raised separately under identical conditions and randomly sampled for histological analysis.

Triploid males underwent testicular development and proliferation of germ cells by mitosis and meiosis, progressing through initial phases of spermatogenesis at a similar pace to diploid controls. The effects of triploidy on males were most evident during the final stages of spermatogenesis, when all diploid males contained free spermatozoa in the lumen of most tubules (Average Relative Frequency, ARF= 68.5%), whereas triploid males contained predominantly spermatocytes (ARF= 36.3%) and morphologically abnormal spermatozoa (ARF= 31.8%).

In contrast, the gonadal development of triploid females was affected during its early stages; the major patterns observed being the arrest of the oogonia within oogonial clusters (ARF= 30.4-71.1%), the appearance of small numbers (ARF= 1.5-6%) of previtellogenic and early vitellogenic follicles, and the proliferation of non-follicular elements (i.e. *vascular lacunae*, fibrosis, tubular adenomas). A major finding was the presence of male-differentiating areas in most triploid females examined, which by the end of the sampling period appeared as gonadal hermaphrodites. It is hypothesised that the lack of proper somatic-to-germ cells interactions prevents the segregation of the oocytes from the gonial clusters and may explain the early blockage observed during the gonadal morphogenesis of autotriploid female rainbow trout.

4.2 Introduction

Ploidy manipulation in fish has become an important commercial tool in recent years, centred on the production of triploid individuals (Chourrout, 1980; Wolters *et al.*, 1982). The main goal of producing triploid fish is their potential sterility, which is assumed to arise from the triploid genome of germ cells leading to aberrant disjunction at anaphase I and resulting in the suppression of gametogenesis (Thorgaard and Gall, 1979). The production of sterile fish is of interest to the fish farming industry as a way of preventing the undesirable effects concomitant with sexual maturation in many teleosts, particularly salmonids (Utter *et al.*, 1982; Thorgaard, 1986; Ihssen *et al.*, 1990; Hussain, 1996). The reduction in growth resulting from a massive energetic investment in gonadal development (up to 10% of their body weight in males and 30% in females), the deterioration of flesh quality during maturation and the high mortality at the time of spawning can be cited among the deleterious effects that sexual maturation brings about in farmed stocks (Lincoln and Scott, 1984; Bye and Lincoln, 1986; Pillay, 1993). Ploidy manipulation is now also regarded as a useful strategy for preventing the loss of biodiversity resulting from genetic introgression caused from escaped farm fish in areas of intensive aquaculture (Johnstone, 1995).

However, in spite of considerable commercial interest in the use of triploid stocks, their reproductive development remains poorly understood. Evidence to date indicates that the reproductive effects of induced triploidy vary in different fish species and sexes, from complete or partial sterility to functional reproduction. In the rainbow trout, as in other salmonids, there seems to be a marked difference in the effects of triploidy on the gonadal development of males

and females. Triploid males show an endocrine profile similar to diploid males, and their germ cells enter meiosis with no obvious delay in comparison to diploid males, although subsequent spermatogenesis appears to be substantially delayed (Kobayashi *et al.*, 1993) resulting in the production of low numbers of morphologically abnormal, aneuploid spermatozoa (Benfey *et al.*, 1986). By contrast, steroid hormone levels remain remarkably low in triploid females of up to 27 months of age (Nakamura *et al.*, 1987). The ovaries of triploid females retain a string-like appearance, although low numbers of vitellogenic oocytes have been observed in triploid rainbow trout (Lincoln, unpublished, cited in Lincoln and Scott, 1984; Okada, 1985) and other triploid salmonids (Johnson *et al.*, 1986; Benfey, 1995).

The majority of previous reports on the reproductive characteristics of triploid rainbow trout have focused on the gonadal histomorphology and steroid profiles at the time of their first sexual maturation, rather than on providing a detailed account of gametogenesis throughout their life cycle. Presented in this study are the results of a long term, quantitative analysis aimed to (1), obtain an accurate description of sexual differentiation and gametogenesis in these ploidy-manipulated animals, (2) to quantify their degree of germ line development and reproductive potential, and (3) to investigate possible histopathological aberrations arising during the gonadal development of male and female triploid rainbow trout.

4.3 *Materials and Methods*

4.3.1 *Animals*

Triploid rainbow trout were produced from broodstock selected from crosses of RTJ ('Javier') and RTD ('Davis') Mt. Lassen-derived strains (2 year-old, 1.2-1.5 kg body weight). Pooled eggs stripped from three mature females were fertilised with sperm collected from four different males (water temperature = 13.6 °C). Triploidy was induced by a 13-min, 27 °C heat shock applied to eggs 11 min after fertilisation to induce the retention of the second polar body. Eggs were then incubated at 13 ± 2 °C for 26 days, when hatching took place. Ploidy was determined by measuring the nuclear size of erythrocytes with a Coulter Counter and Channeliser (Coulter Instruments, Colorado, USA)(Wattendorf, 1986). Triploid and diploid (control) siblings were raised in separate tanks at similar density, feeding and water quality regimes (T= 10-18 °C, ambient photoperiod).

4.3.2 *Experimental Design*

Triploid and diploid groups were sampled at 7, 15, 20, 25 and 44 months post-hatching by over-anaesthetising randomly selected individuals in a 0.01% solution of benzocaine. After ploidy reassessment by Coulter Counter, a separate sample was collected from the anterior, median, and posterior parts of the gonads when size made it possible, and fixed in 10% buffered formalin. Replicate sections (thickness = 5-6 μ m) were prepared from a total of 346 paraffin-embedded tissue blocks, and stained with haematoxylin-eosin, the periodic acid-Schiff reaction, Heidenhain's iron haematoxylin and Masson's trichrome (Carleton, 1980). Slides were examined under low power magnification (4X to 40X) with an Olympus BH-2 microscope

(Olympus Optical Co. Ltd., Japan) linked to a Sony colour monitor *via* a Panasonic F10 videocamera (Matsuhita Co. Ltd., Japan). Three different fields were examined per section in order to ensure random sampling. A 'short line' multipurpose grid (Graticules Ltd., England) was placed in an eyepiece between the microscope and the videocamera, the image of the grid overlaying the gonadal section and also visible on the monitor. The total number of grid points on the monitor and the number of grid points lying within a given cell stage or category were recorded, and Relative Frequencies (RFs) for a given cell category computed as:

$$\text{Relative Frequency} = \frac{\text{Grid points laying within cell category}}{\text{Total number of grid points}} \times 100$$

Gonadal cell stages were classified according to previous morphological studies on gametogenesis in the rainbow trout (Billard, 1986, 1992; Bromage and Cumaranatunga, 1988; see Sections 1.7.3 and 1.7.6 of this Thesis).

With the exception of the first sampling, sex was easily ascertained for all sections according to the general morphology and staining affinity of the gonad. The main morphological feature to establish sex at 7 months of age was the appearance of the characteristic lamellar structure present in female teleosts, since in the majority of the individuals sampled at this time the lobular arrangement typical of males was still not established. Thus, individuals with gonads displaying the lamellar structure at this age were classified as females, and the remaining individuals were assumed to be males. A summary with the number and sex of individuals analysed at every sampling time for both ploidy groups is presented in Table 4.1.

Months	Males		Females	
	Diploid	Triploid	Diploid	Triploid
7	11	9	11	11
15	13	11	8	9
20	4	4	6	6
25	9	10	10	10
44	1	1	5	10
TOTAL	38	35	40	46

Table 4.1. Sample size and observed gonadal sex in diploid and triploid groups at different age stages.

4.3.3 Statistical Analysis

Relative Frequencies were averaged for each fish in order to obtain the Individual Relative Frequencies (IRFs). Individual Relative Frequencies were averaged for each ploidy group to calculate the Average Relative Frequencies (ARFs) at every sampling time. Average Relative Frequencies were arc-sine transformed and compared between ploidy groups by one-way ANOVA (Sokal and Rohlf, 1981; Zar, 1996). Differences were considered significant at the $P < 0.05$ level.

4.4 Results

4.4.1 Males

4.4.1.1 Gonadal structure at 7 months post-hatching.

In ten of the eleven diploid males sampled at seven months of age the gonad consisted of groups of 5-6 cells at the spermatogonia A and B stages, which appeared cysted in circular arrangement and externally delimited by a thin layer of connective stroma. Typically, spermatogonia type B (ARF= 43.5%, Figure 4.1, Appendix 8) were found in a central position within the cysts and showing mitotic activity, while type A spermatogonia (ARF= 22.4%) were located peripherally. These cysts were surrounded by variable numbers of interstitial (Leydig) cells (ARF= 14.2%) characterised morphologically by flat, scarce cytoplasm and darkly staining spheroidal nuclei, located in close contact with the external wall of the cysts. In the margins of the gonad the cystic arrangement was not present, but instead spermatogonia (generally of type A) were in close juxtaposition with interstitial cells, eosinophilic fibres and myeloid-resembling cells (Figure 4.2).

One precocious diploid male displayed a more advanced gonadal morphology characterised by the establishment of the lobular arrangement in which primary spermatocytes (IRF= 53.1%) were embedded in Sertoli cells (IRF= 1.1%) delimited by thin trabeculae of connective stroma. A few scattered lobules showed signs of meiotic activity with secondary spermatocytes (IRF= 22%) and spermatids (IRF= 16.7%) being located in the central area of the lobules.

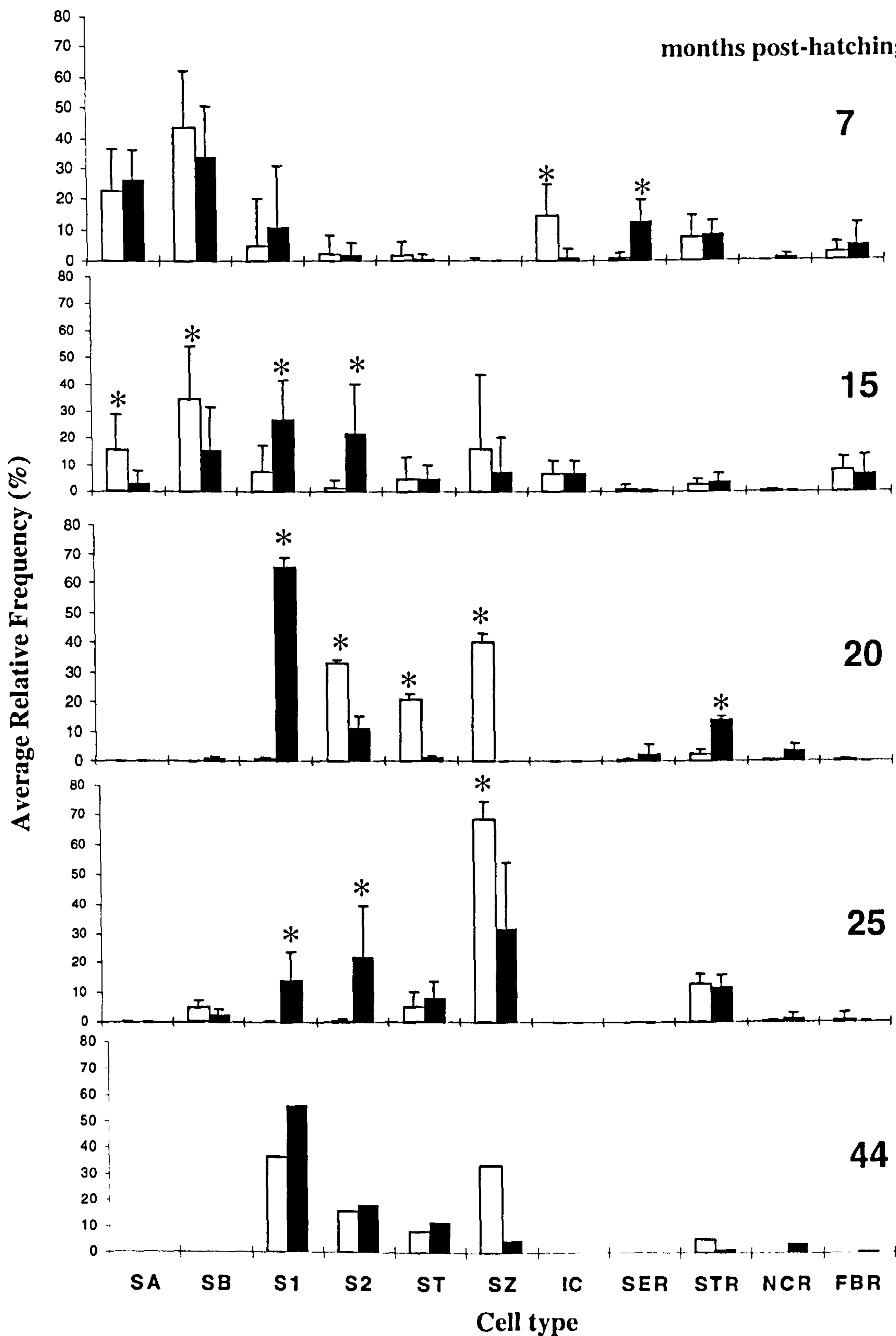


Figure 4.1. Average relative frequencies of gametogenic cells and non-parenchymal components in diploid (□) and triploid (■) rainbow trout testes at different age stages. Bars represent standard deviations, asterisks denote significantly different means ($P < 0.05$). SA, type A spermatogonia; SB, type B spermatogonia; S1, primary spermatocytes; S2, secondary spermatocytes; ST, spermatids; SZ, spermatozoa; IC, interstitial cells; SER, Sertoli cells; STR, stroma; NCR, necrosis; FBR, fibres.

Most males in the triploid group presented a similar anatomy of the gonad, although showing less abundant spermatogonial cysts, more prominent Sertoli cells and a significantly lower number of interstitial cells than diploid males (ARF= 0.9%, Figures 4.1 and 4.3). In the margins of the gonad uncysted spermatogonia were found in close contact with scarce interstitial cells and eosinophilic fibres. Four out of the nine triploid males sampled showed signs of progress through spermatogenesis. These exhibited the nested arrangement of the spermatogonia previously observed in diploid males, with primary spermatocytes (ARF= 10.5%) in the centre of some cysts. In one precocious triploid male the lobular arrangement was evident, with spermatogonia type B in the periphery of the lobules and primary and secondary spermatocytes more centrally positioned. Some spermatids (IRF= 5.2%) with irregular, uneven profiles were present in the lumen of a few lobules.

4.4.1.2 Gonadal structure at 15 months post-hatching.

The testes of ten diploid males at 15 months of age were organised in the lobular arrangement characteristic of salmonid males (Figure 4.4). The lobules appeared filled with spermatogonia type B (ARF= 34.5%) and a few primary spermatocytes (ARF= 7.5%) embedded in Sertoli cells and loosely positioned around a central lumen. Thin trabeculae of connective stroma delimited the lobules. Interstitial cells, eosinophilic fibres and blood vessels with erythrocytes and myeloid-resembling cells were located in the margins of the gonad. Three diploid males contained morphologically normal spermatozoa in the centre of most lobules and spermatids in the periphery (IRFs= 63-73% and 4-8%, respectively).

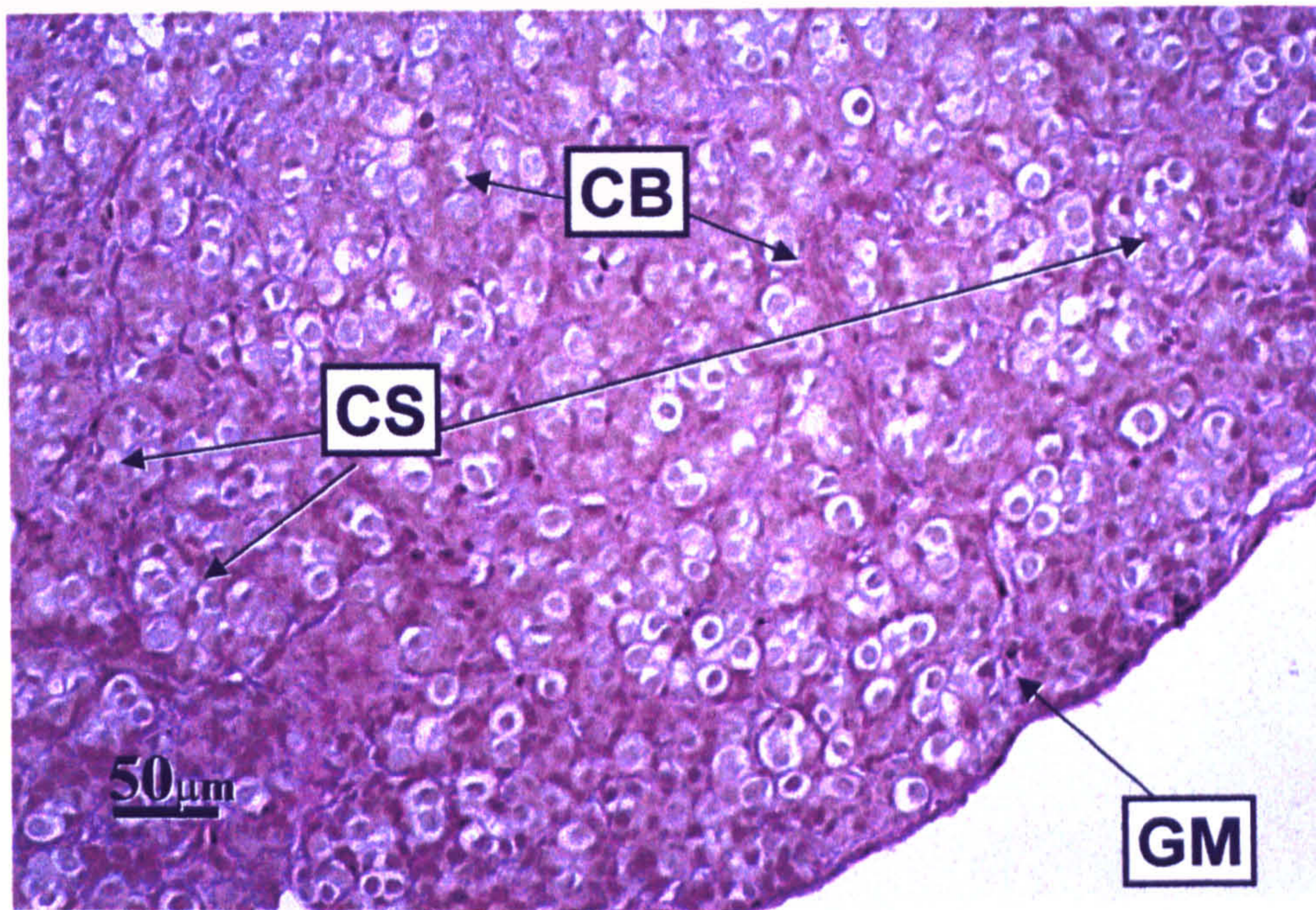


Figure 4.2. Gonadal photomicrograph from a 7 month-old diploid rainbow trout male stained with the periodic acid-Schiff reaction and counterstained with Heidenhain's haematoxylin. CB, cyst boundary; CS, cysted spermatogonia; GM, gonadal margin.

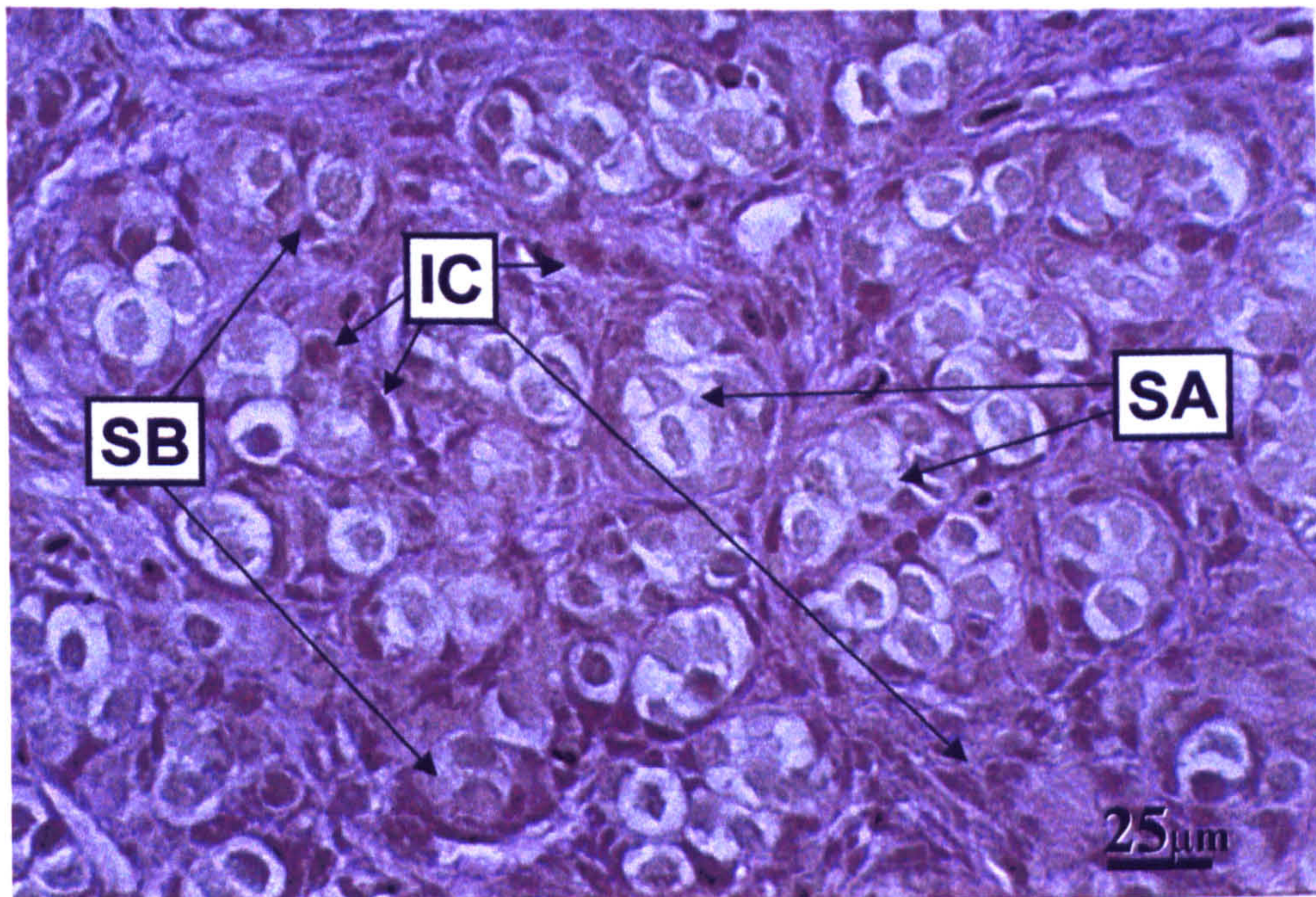


Figure 4.3. Gonadal photomicrograph from a 7 month-old triploid rainbow trout male stained with the periodic acid-Schiff reaction and counterstained with Heidenhain's haematoxylin. IC, interstitial cells; SA, spermatogonia type A; SB, spermatogonia type B.

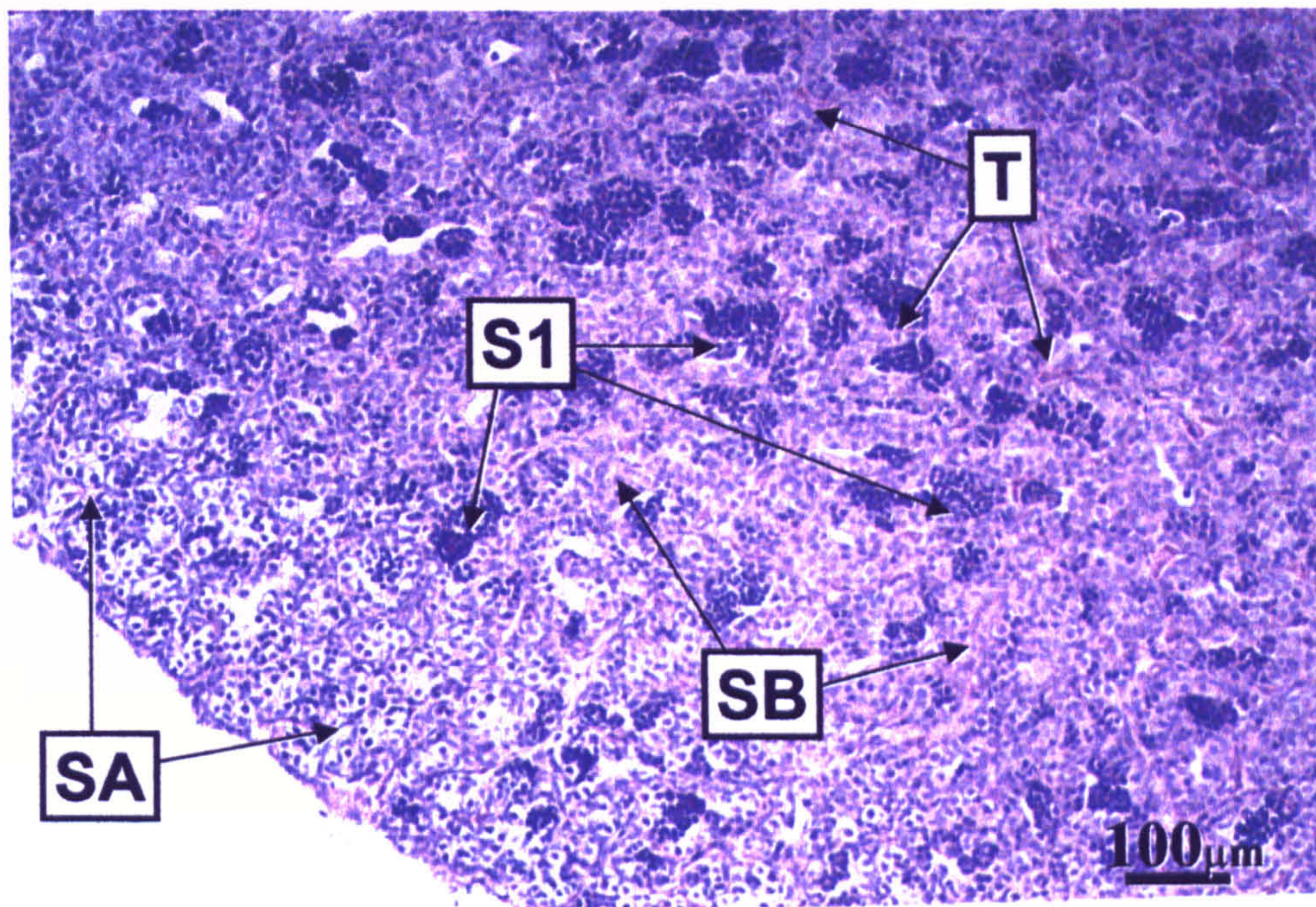


Figure 4.4. Gonadal photomicrograph from a 15 month-old diploid rainbow trout male stained with haematoxylin-eosin. SA, spermatogonia type A; SB, spermatogonia type B; S1, primary spermatocytes; T, trabeculae.

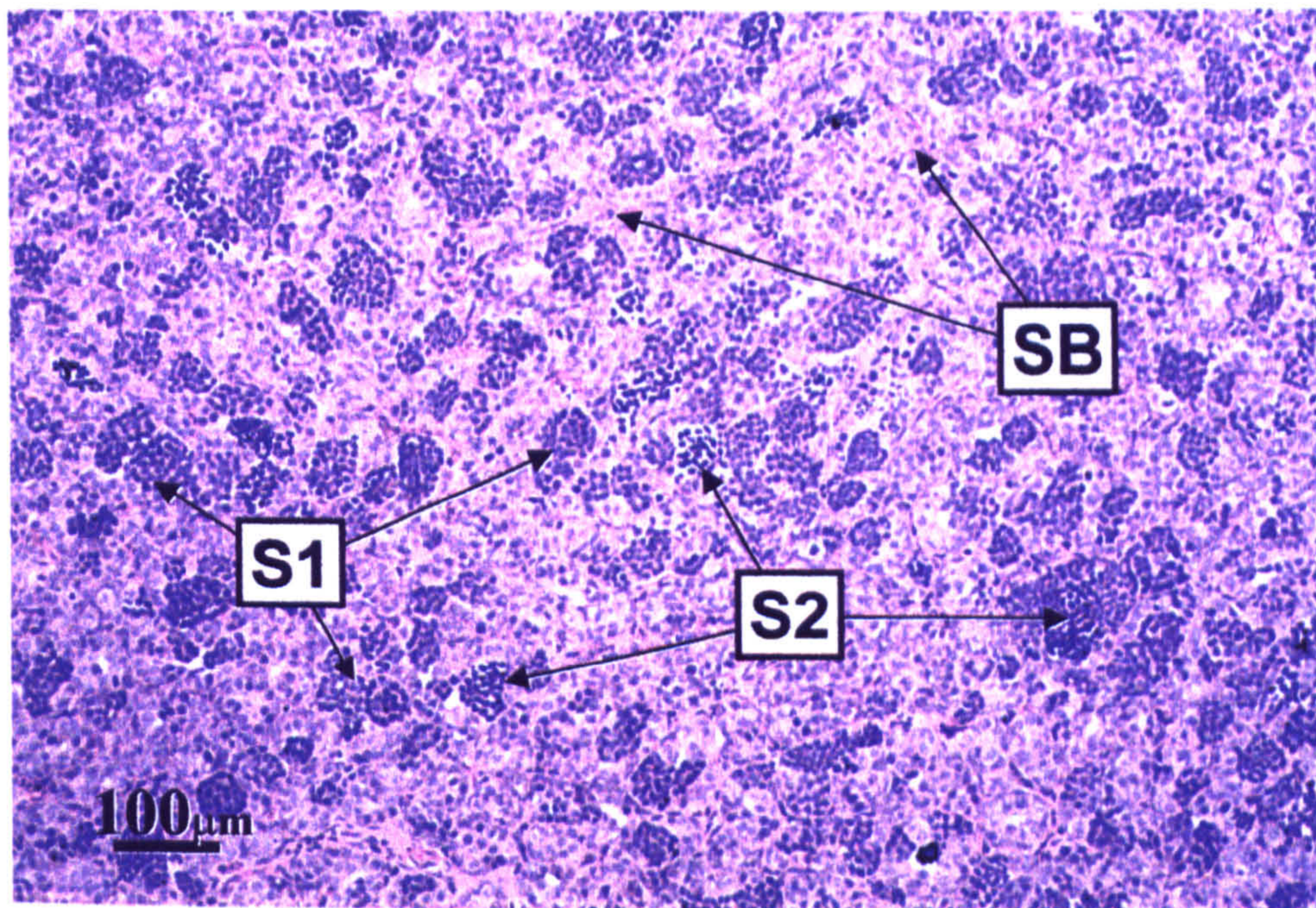


Figure 4.5. Gonadal photomicrograph from a 15 month-old triploid rainbow trout male stained with haematoxylin-eosin. SB, spermatogonia type B; S1, primary spermatocytes; S2, secondary spermatocytes.

In these three fish spermatogonia and interstitial cells were visible within the trabeculae, which appeared thicker than in the previous males. Fibres and erythrocytes were also visible within the trabeculae. Free spermatozoa were present in the lumen of their spermatic ducts. The duct was lined by a single layer of ciliated epithelium, with large blood vessels running through its wall.

The testes of triploid males were also organised in lobules, but contained significantly lower numbers of spermatogonia and showed further development in spermatogenesis than diploid males (Figures 4.1 and 4.5). All eleven sampled individuals had entered meiosis, with the bulk of most lobules being filled with significantly higher numbers of primary (ARF= 26.5%) and secondary spermatocytes (21.3%) than diploid males at this age (Figure 4.6). Spermatocytes had irregular shapes and in many cases their cytoplasm appeared vacuolated. When present, spermatids (ARF= 4.6%) were located in the central lumen of the lobules, showing very heterogeneous size and shape and frequently concomitant with cell debris. Intercellular spaces appeared filled by a basophilic substance reminiscent of colloid. The trabeculae delimiting the lobules appeared thicker than in diploid males and contained interstitial cells (ARF= 6.5%). The margins of the gonads contained some spermatogonia type A (ARF= 2.9%) associated with eosinophilic structures resembling collagen fibres. Five of the eleven triploid males examined had variable proportions of spermatozoa (IRFs= 0.7-41.3%) present in the central lumen of most lobules. Size and shape of spermatozoa was also variable among the lobules, although in some cases they exhibited homogeneous morphology and visible flagella. In one of these males most lobules were filled with normal-looking spermatozoa (IRF= 41.3%) while some appeared empty after

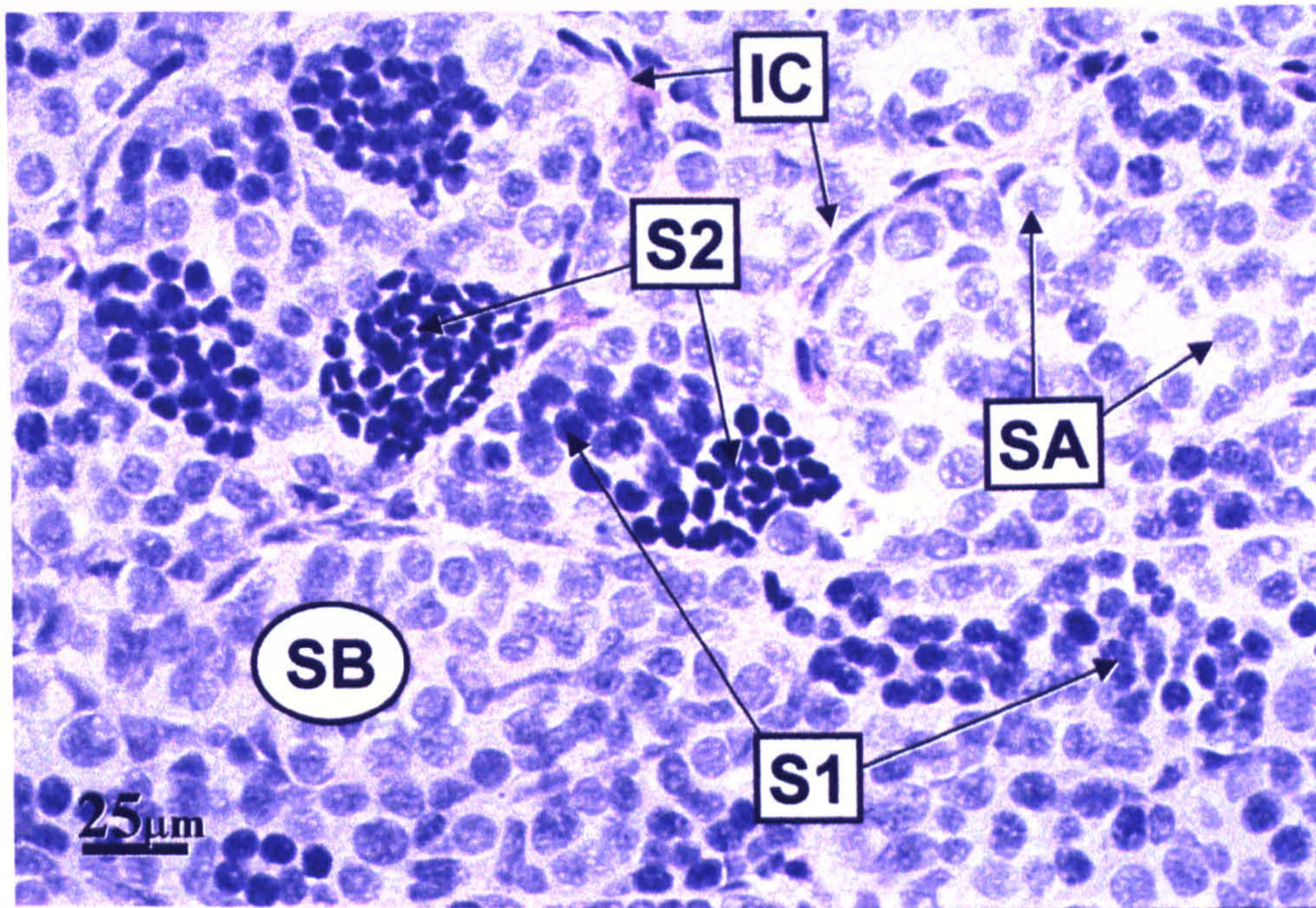


Figure 4.6. Gonadal photomicrograph from a 15 month-old triploid rainbow trout male stained with haematoxylin-eosin. IC, interstitial cells; SA, spermatogonia type A; SB, spermatogonia type B; S1, primary spermatocytes; S2, secondary spermatocytes.

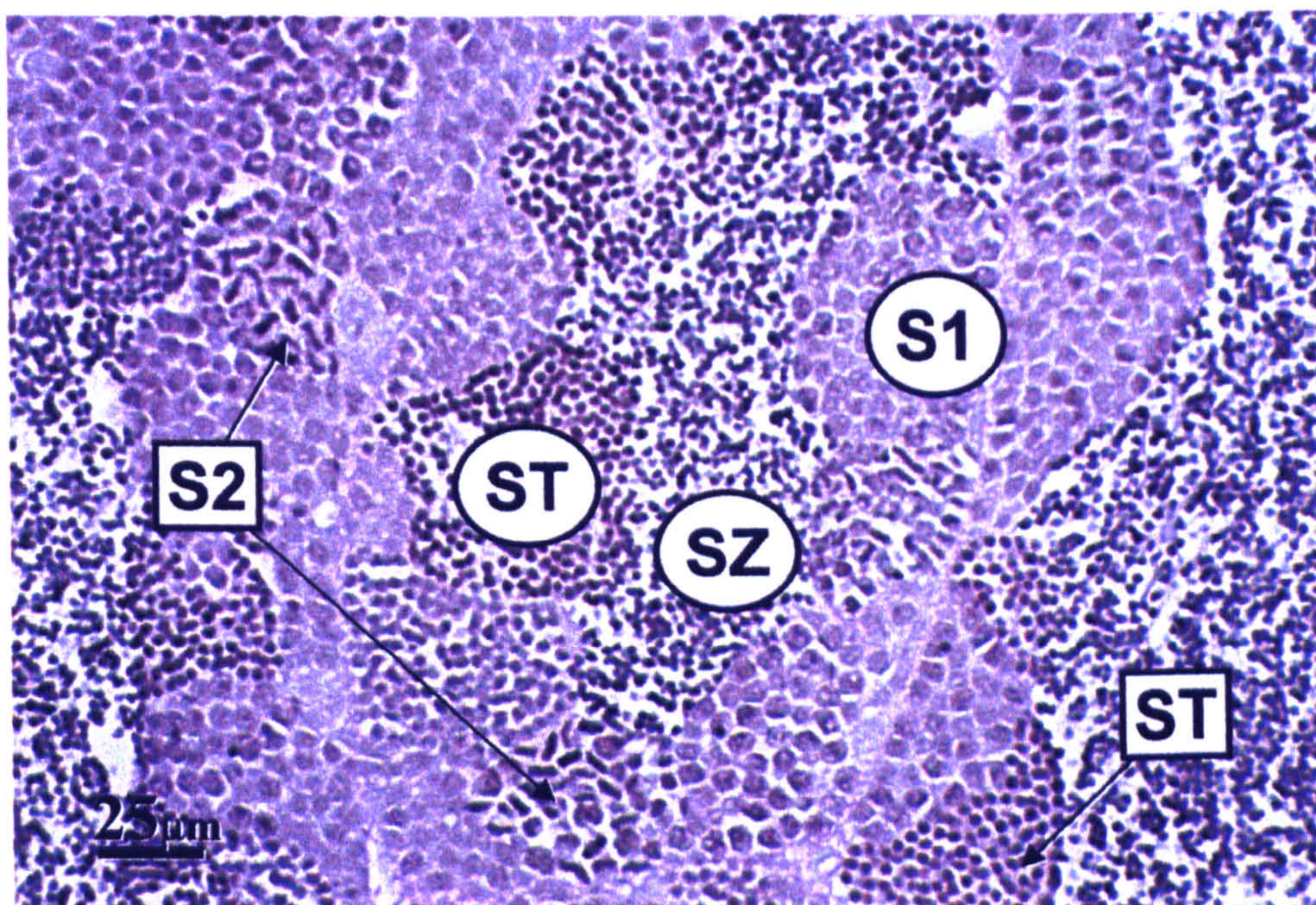


Figure 4.7. Gonadal photomicrograph from a 20 month-old diploid rainbow trout male stained with the periodic acid-Schiff reaction and counterstained with Heidenhain's haematoxylin. S1, primary spermatocytes; S2, secondary spermatocytes; ST, spermatids; SZ, spermatozoa.

apparent spermiation, delimited by thickened trabeculae containing primary spermatocytes embedded in Sertoli cells. In this male, spermatozoa were present in the lumen of the spermatic duct, which was lined by ciliated epithelium and highly vascularised.

4.4.1.3 Gonadal structure at 20 months post-hatching.

All four diploid males were completing spermiogenesis at the age of 20 months, showing variable numbers of fully differentiated spermatozoa in the lumen of their lobules. Secondary spermatocytes (ARF= 32.7%) and spermatids (ARF= 20.8%) were observed in a marginal position within the lobules (Figure 4.7). Free spermatozoa (ARF= 39.8%) presented regular, even morphology and homogeneous size across the lobules. An extracellular basophilic substance was also present in the lumen of these lobules.

In triploid males, the stromal component delimiting the testicular lobules appeared significantly more prominent than in diploid males at this age, and showing intense vascularization. Most cells within the lobules were at the stage of primary spermatocytes (ARF= 65.1%) with vacuolised cytoplasm, although some secondary spermatocytes (ARF= 11.1%) and very few spermatids (ARF= 1.1%) were also present. These, however, appeared in significantly lower numbers than in diploid males (Figure 4.1). The central lumen of the lobules contained filamentous, thread-like structures and cell debris. No spermatozoa were present in the lumen of the spermatic duct (Figure 4.8).

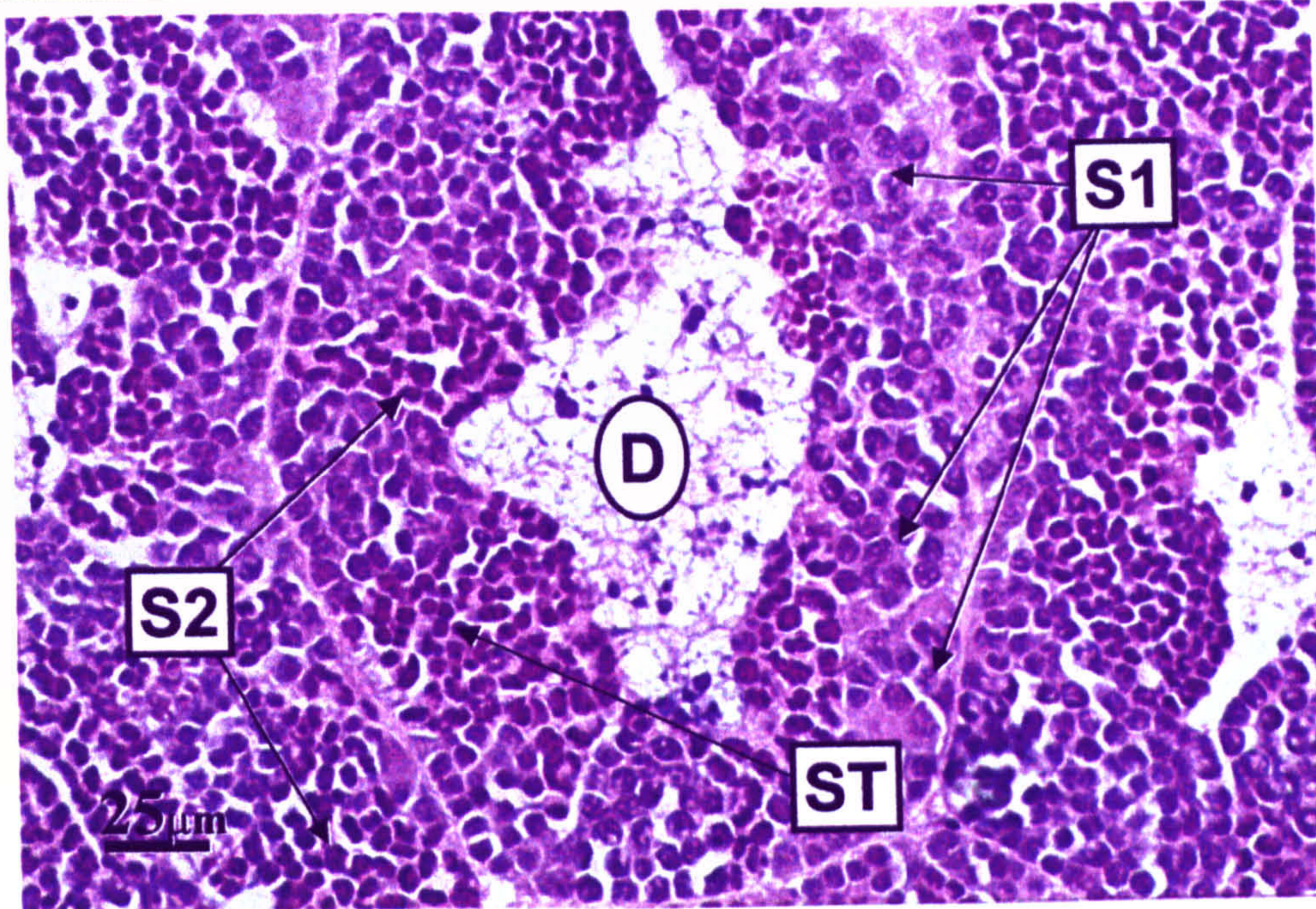


Figure 4.8. Gonadal photomicrograph from a 20 month-old triploid rainbow trout male stained with haematoxylin-eosin. D, cell debris; S1, primary spermatocytes; S2, secondary spermatocytes; ST, spermatids.

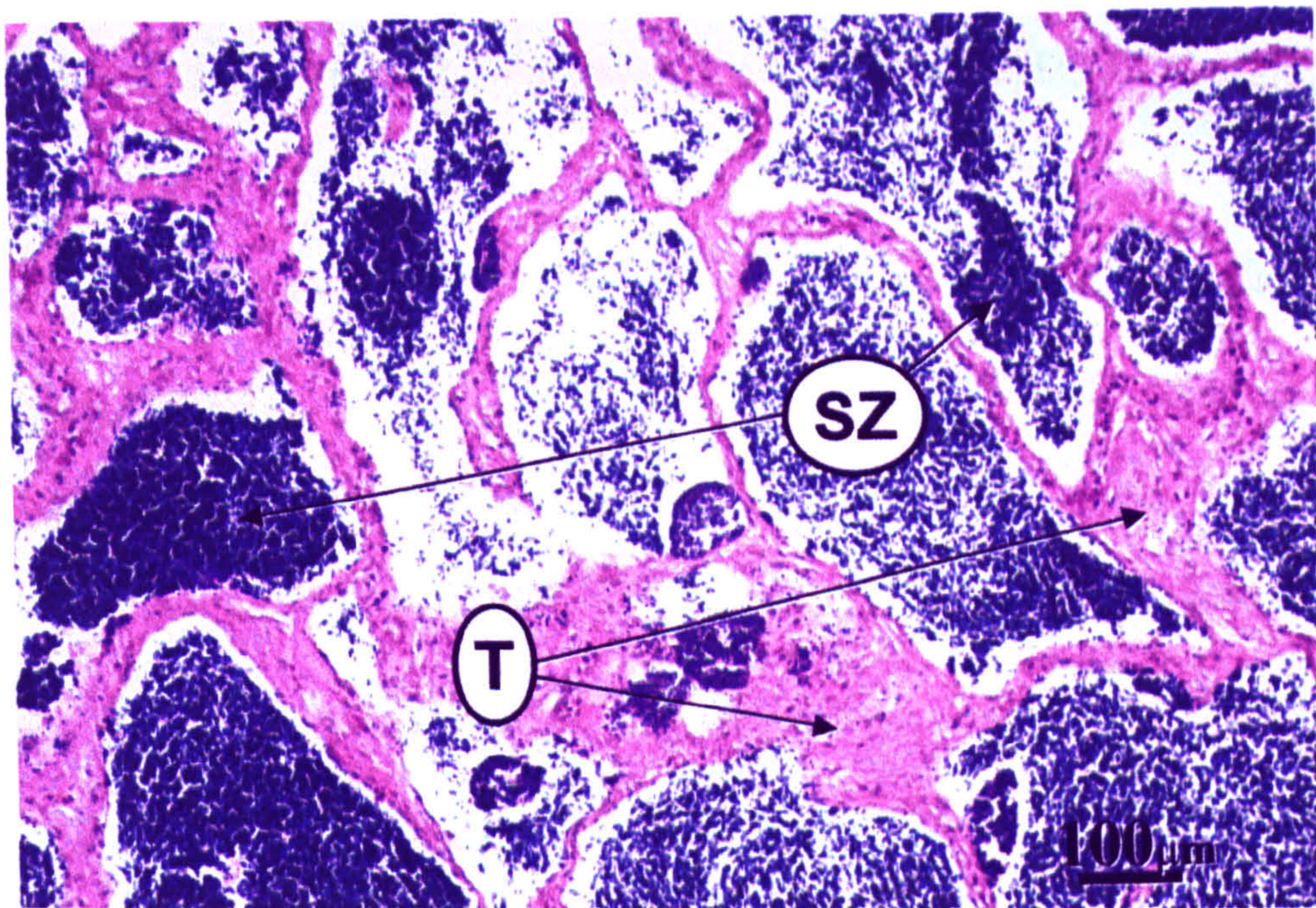


Figure 4.9. Gonadal photomicrograph from a 25 month-old diploid rainbow trout male stained with haematoxylin and eosin. SZ, spermatozoa; T, trabeculae.

4.4.1.4 Gonadal structure at 25 months post-hatching.

All diploid males had spermiated at 25 months of age (Figure 4.9). The central area of many testicular lobules appeared as an empty space, while in the proliferating stroma, type B spermatogonia (ARF= 4.4%) and very few spermatocytes (ARF= 0.4%) were observed among a relatively high proportion of fibres (ARF= 0.9%). Lobules which still contained free spermatozoa in their lumen (ARF= 68.5%) showed signs of degeneration such as cell agglutination and overstaining, and a basophilic substance filled the intercellular spaces. Some residual spermatozoa were visible in the spermatic duct of all ten males sampled.

The majority of triploid males, on the contrary, were still completing spermiogenesis, with significantly higher proportions of spermatocytes and lower numbers of spermatozoa than diploid males (Figure 4.1). There seemed to be some overlap between the first and second gonadal cycles in the ten triploid males examined at this age. Typically, abnormal spermatozoa (ARF= 31.8%), spermatids (ARF= 8.1%) and secondary spermatocytes (ARF= 22.1%) were found in the centre of the lobules together with cell debris and colloid. The lobules were delimited by proliferating stroma containing type B spermatogonia (ARF= 2.3%) and primary spermatocytes (ARF= 14.2%). Spermatozoa and spermatids presented irregular morphology and variable cell size, and cytoplasmic vacuolisation was evident in secondary spermatocytes. Three of the ten triploid males, however, exhibited uniform and morphologically-normal free spermatozoa (IRFs= 48-69%) in the lumen of most lobules (Figure 4.10). In two of these cases normal-looking spermatozoa were also found in the lumen of the spermatic duct, which was lined by a single layer of ciliated epithelium (Figure 4.11).

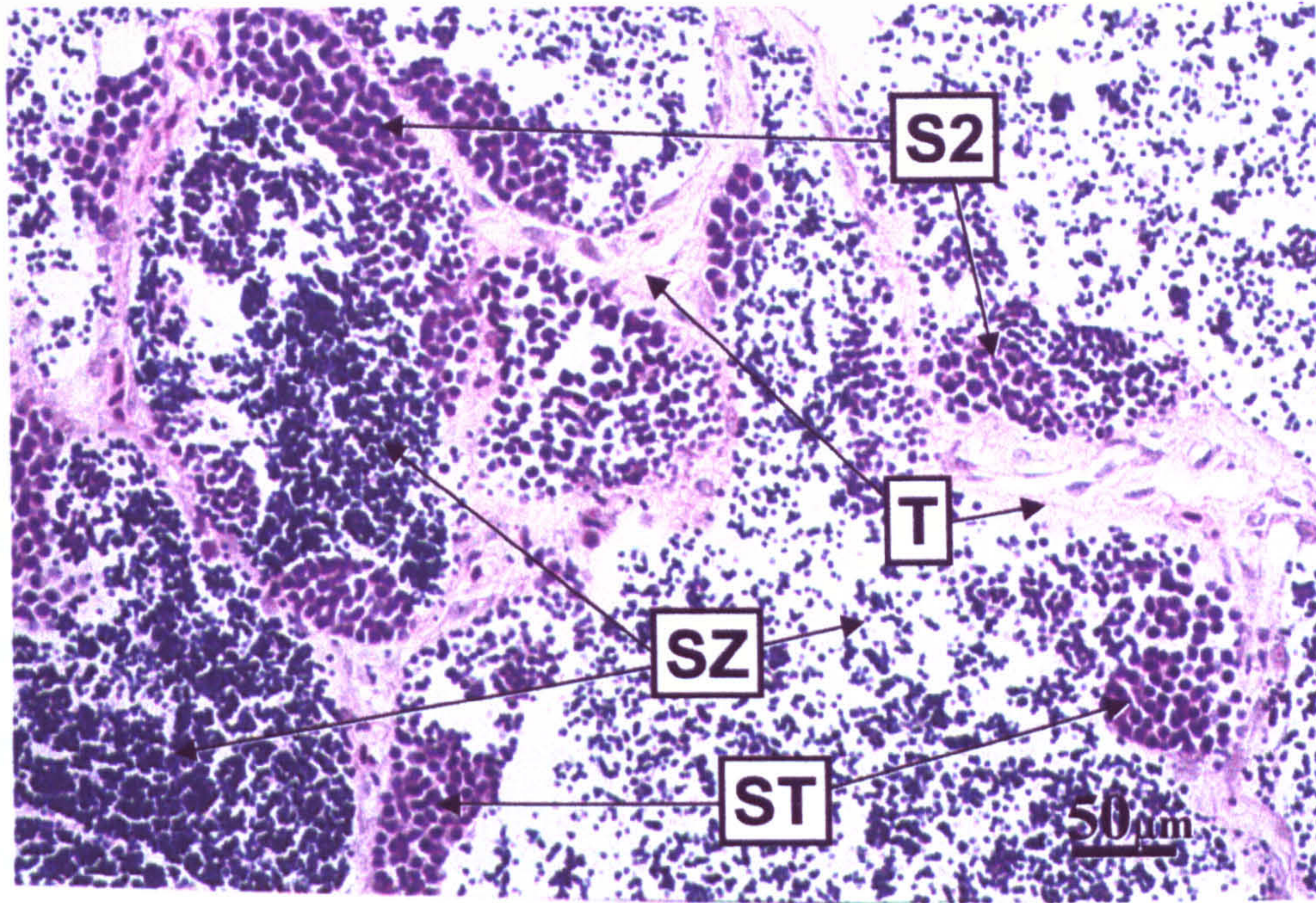


Figure 4.10. Gonadal photomicrograph from a 25 month-old triploid rainbow trout male stained with haematoxylin and eosin. S2, secondary spermatocytes; ST, spermatids; SZ, spermatozoa; T, trabeculae.

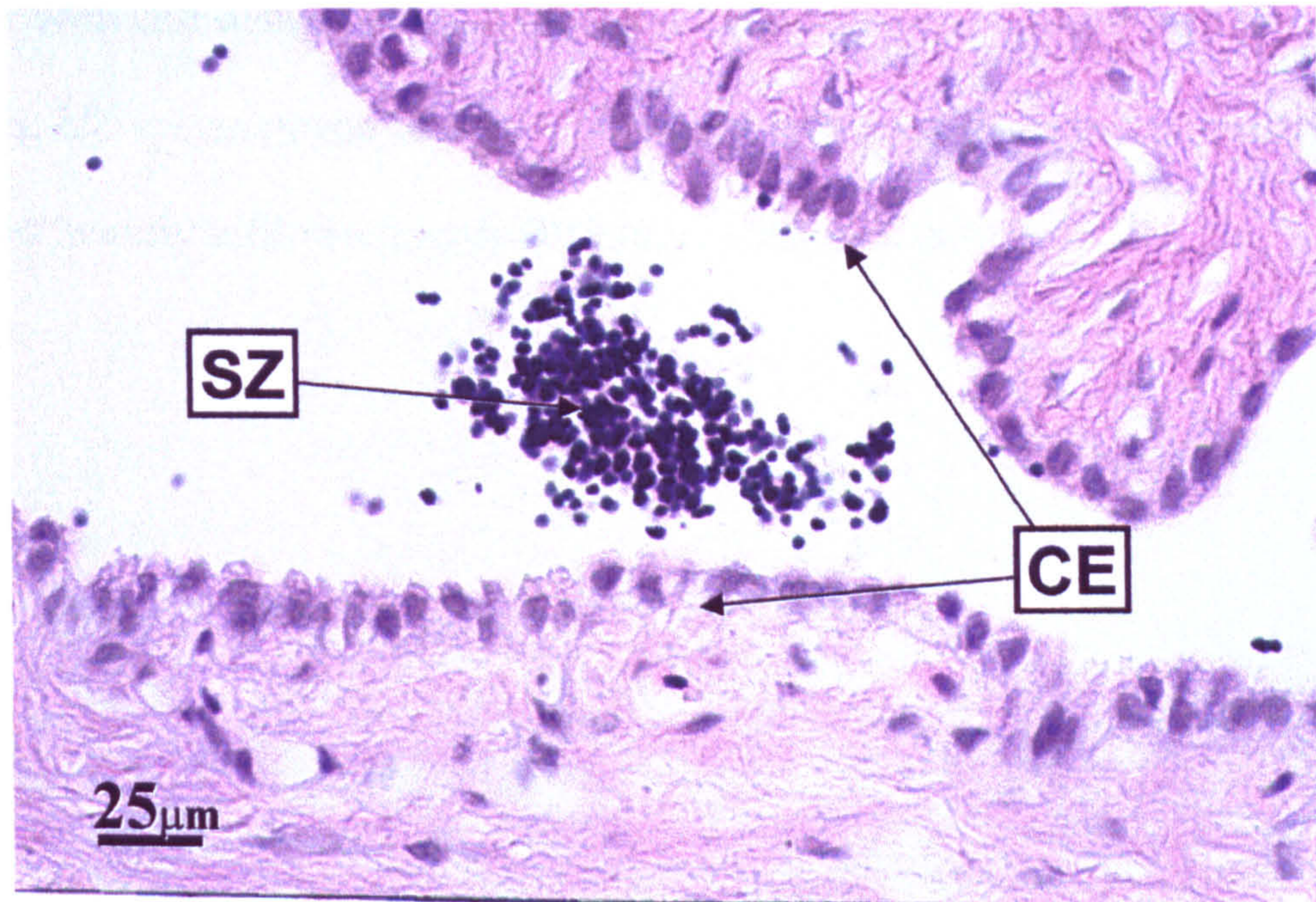


Figure 4.11. Photomicrograph of the spermatic duct of a 25 month-old triploid rainbow trout male stained with haematoxylin and eosin. CE, ciliated epithelium; SZ, spermatozoa.

4.4.1.5 Gonadal structure at 44 months post-hatching.

In the only diploid male available for histological analysis at 44 months of age, the testis had completed a new cycle and progressed to the stage of spermiogenesis. Free spermatozoa (IRF= 33.3%) were visible in the lumen of most lobules, while spermatids were located peripherally. Meiotic activity was still evident in some lobules where primary (IRF= 36.4%) and secondary spermatocytes (IRF= 15.6%) appeared concomitantly with a few spermatids (IRF= 8.3%). No spermatozoa were visible in the lumen of the spermatic duct. The duct wall presented a heavy fibrous infiltration alternating with areas of fat deposits.

The triploid male contained mostly primary spermatocytes (IRF= 56.1%) entering meiosis with some secondary spermatocytes (IRF= 18.2%) and spermatids (11.8%) in the centre of the lobules. Some lobules also contained scattered spermatozoa (IRF= 4.8%) with very heterogeneous size and cell morphology, together with cell debris. Localised areas of fibrosis were also evident throughout the testis. No spermatozoa were visible in the lumen of the spermatic duct, which appeared heavily infiltrated with fibres and adipose tissue.

4.4.2 Females

4.4.2.1 Gonadal structure at 7 months post-hatching.

In all eleven diploid females examined at 7 months of age the ovary was organised in a series of transverse septa extending along the ovarian axis known as the ovigerous lamellae. The lamellae opened into a central ovarian lumen, with their external surface covered by a single layer of cubical epithelium (germinal epithelium), and the gonad was externally delimited by the peritoneum, continuous with the tunica albuginea. The tunica albuginea consisted of a thin fibrous layer covered externally with ciliated columnar epithelium. The lamellae appeared filled with stage 3 oocytes (ARF= 64.8%, Figure 4.12, Appendix 7) embedded in a loosely bound connective stroma containing scattered oogonia (ARF= 3.2%), stage 1 (ARF= 1.2%) and 2 (ARF= 10.6%) oocytes and thin blood capillaries. Stage 3 oocytes exhibited a basophilic cytoplasm and a central nucleus with several nucleoli lying close to the nuclear envelope. They were surrounded by a single layer of flattened granulosa cells and some squamous thecal cells. A few oocytes had developed cytoplasmic vesicles in the periphery of the ooplasm, thus entering stage 4 (ARF= 0.9%).

All triploid females examined showed a much more rudimentary gonadal anatomy than their diploid counterparts, with non-follicular elements overrepresented in their ovaries (Figure 4.13). Although the lamellar organisation was present in all of them, the central lumen of the ovary appeared disrupted longitudinally as opposite lamellae were fused in bridges running through the width of the organ, giving it a closed appearance (Figure 4.14).

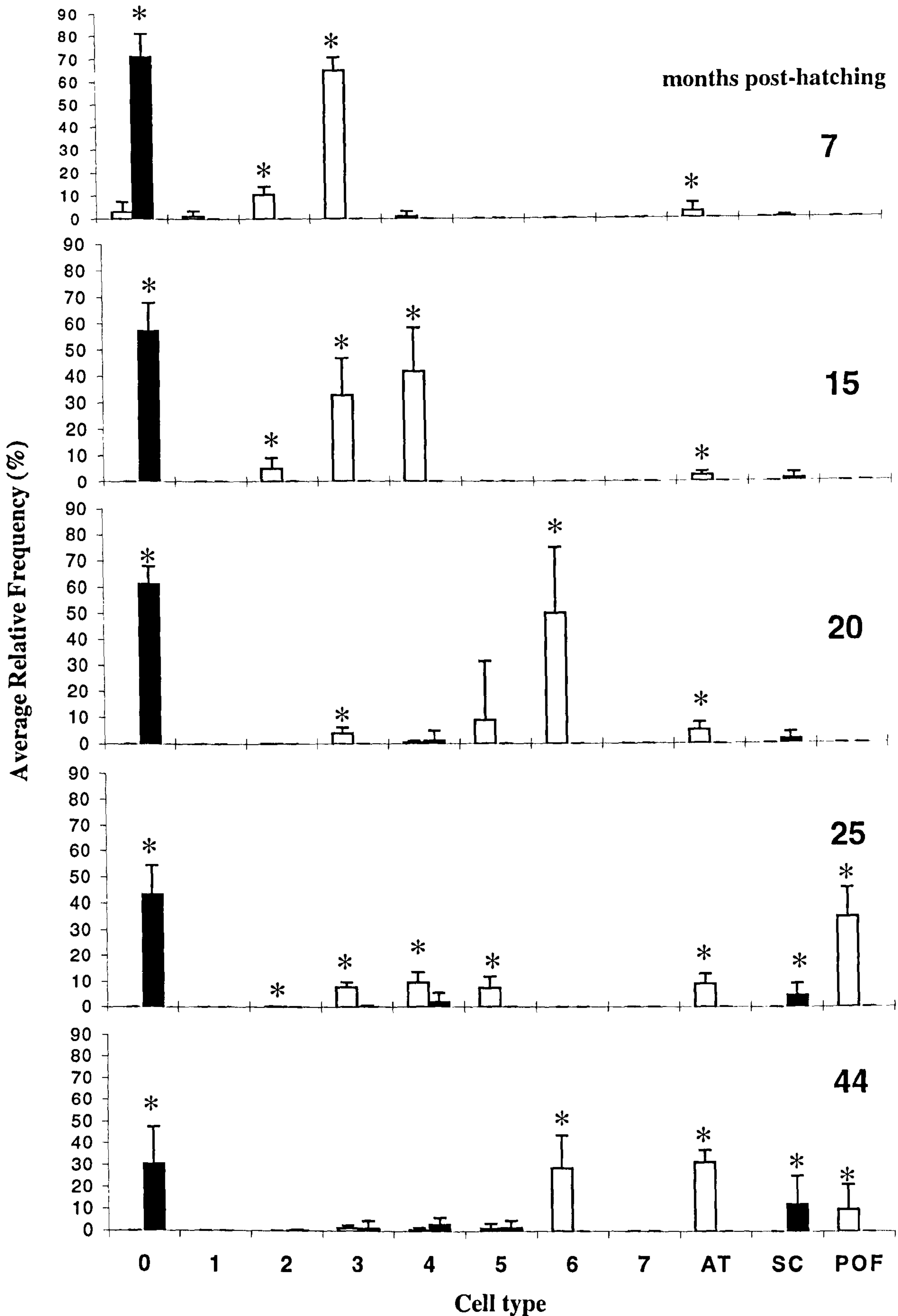


Figure 4.12 Average relative frequencies of gametogenic cells in diploid (□) and triploid (■) rainbow trout ovaries at different age stages. Bars represent standard deviations, asterisks denote significantly different means ($P < 0.05$). 0, oogonia; 1-7, oocyte stages (after Bromage and Cumarantunga, 1988); AT, atretic oocytes; SC, spermatogenic cells; POF, post-ovulatory follicles.

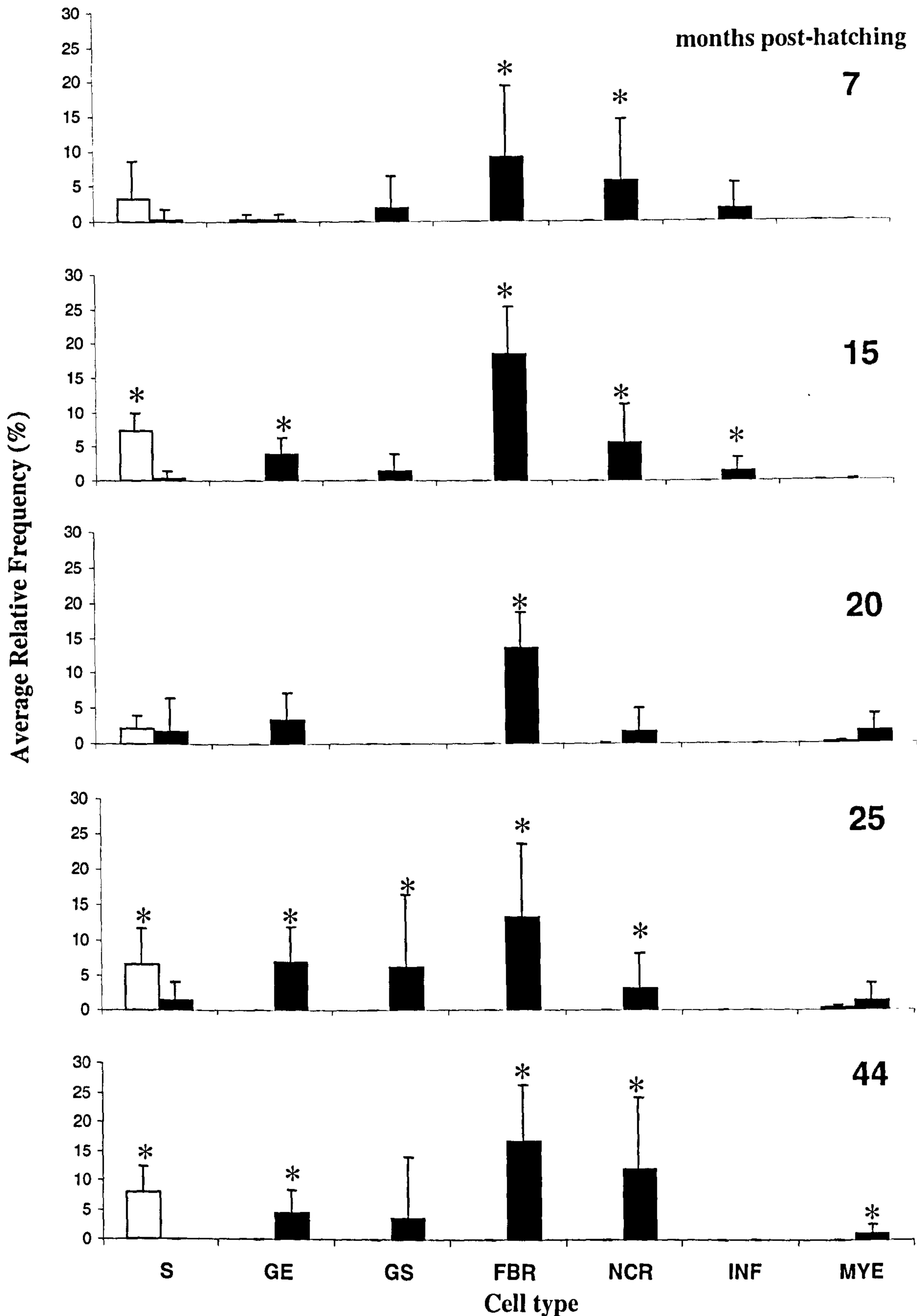


Figure 4.13. Average relative frequencies of non-follicular components in diploid (□) and triploid (■) rainbow trout ovaries at different age stages. Bars represent standard deviations, asterisks denote significantly different means ($P < 0.05$). S, stroma; GE, germinal epithelium; GS, glandular structures; FBR, fibres; NCR, necrosis; INF, inflammatory cells; MYE, myeloid-resembling cells.

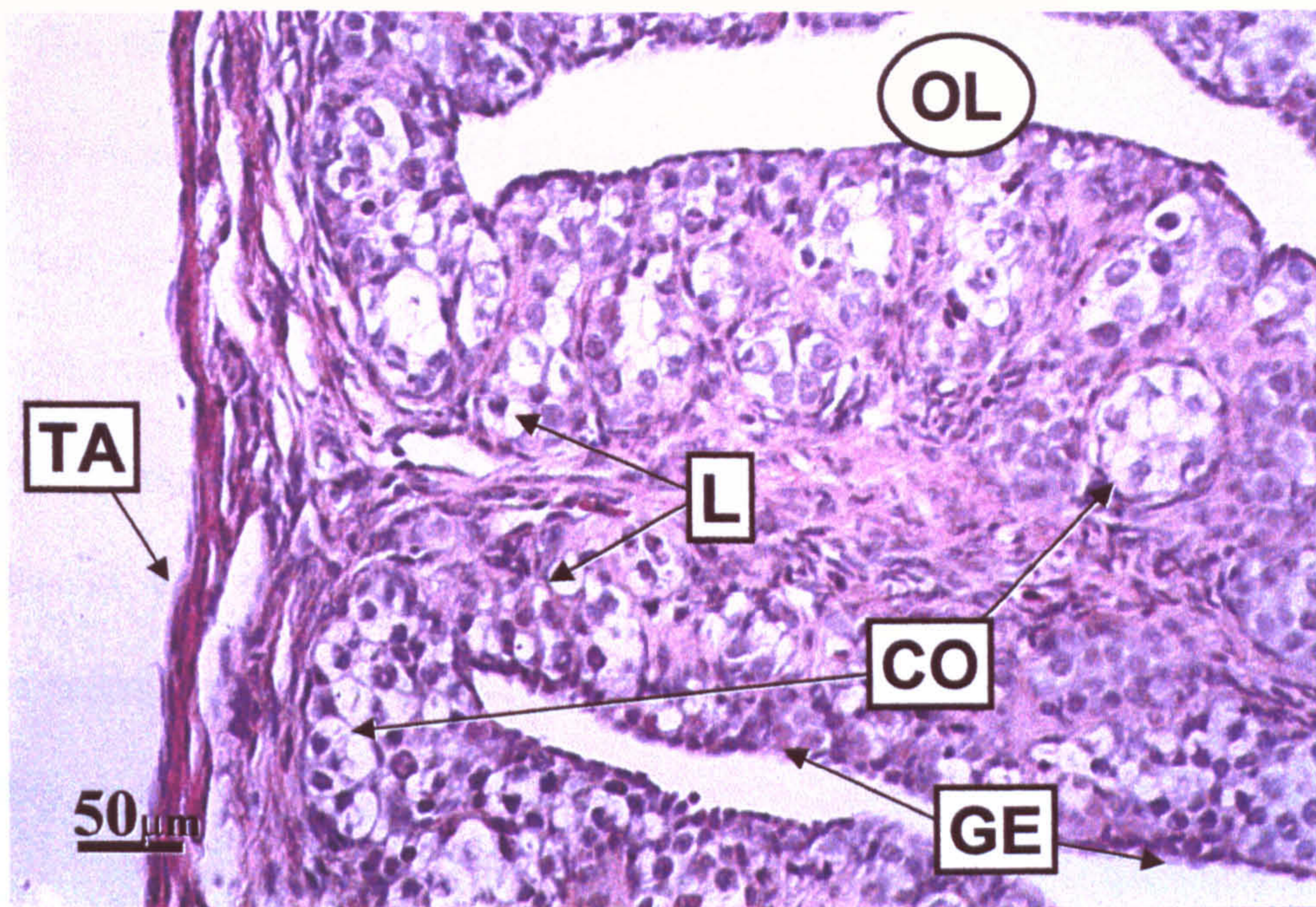


Figure 4.14. Gonadal photomicrograph from a 7 month-old triploid rainbow trout female stained with haematoxylin and eosin. CO, cysted oogonia; GE, germinal epithelium; L, ovarian lamellae; OL, ovarian lumen; TA, tunica albuginea.

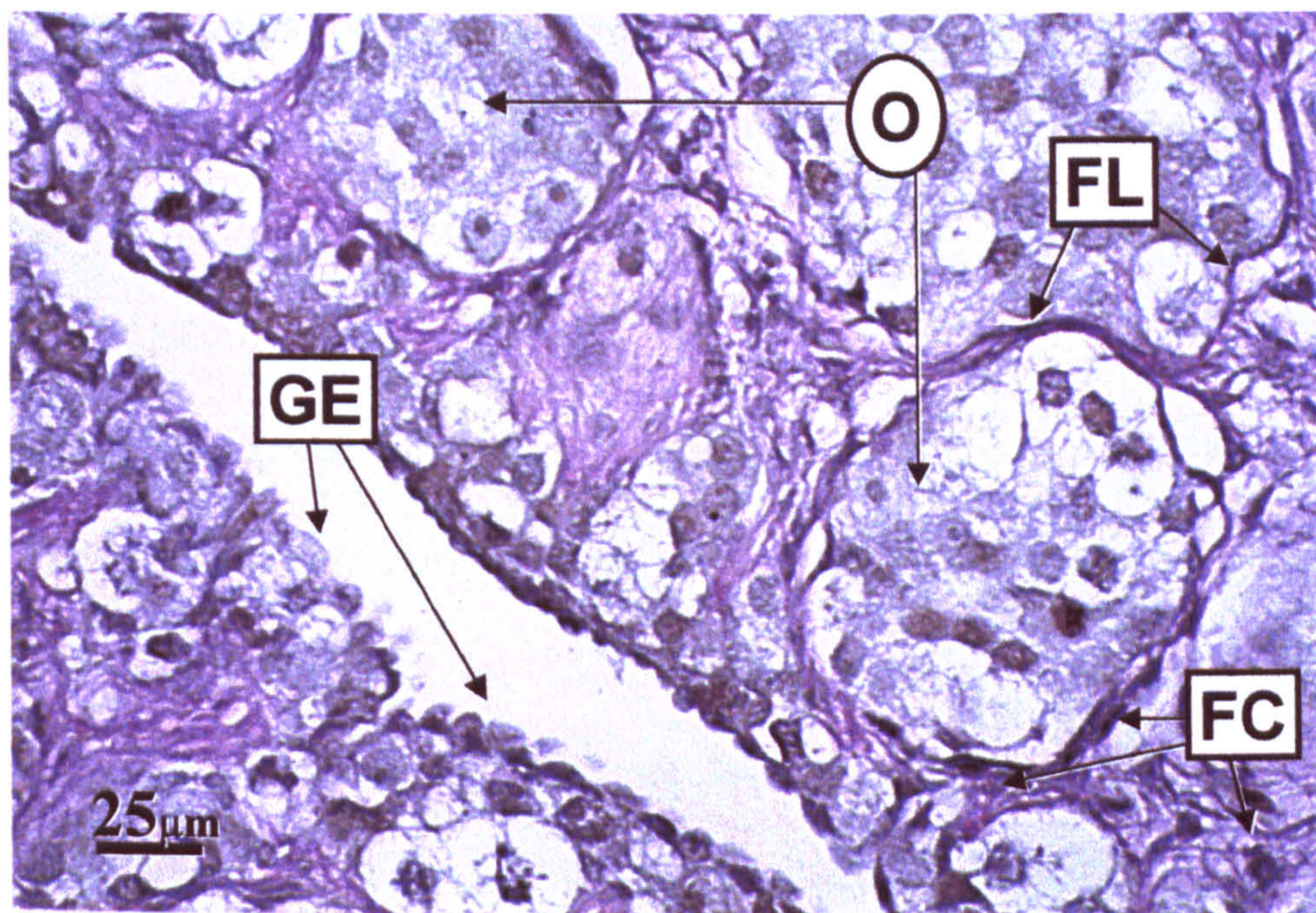


Figure 4.15. Gonadal photomicrograph from a 15 month-old triploid rainbow trout female stained with the periodic acid-Schiff reaction and counterstained with Heidenhain's haematoxylin. FC, prefollicle cells; FL, fibrous layer; GE, germinal epithelium; O, oogonia.

The tunica albuginea appeared greatly thickened by the presence of 5-6 layers of heterogeneous fibrous elements similar to collagen and smooth-muscle fibres intermixed with flattened cells with a central, ellipsoidal nucleus. Germ cells within the lamellae were at the oogonial stage (ARF= 71.1%, Figure 4.12). They had a large spheroidal nucleus positioned centrally, often showing signs of vacuolisation and pyknosis, and a stranded cytoplasm. In 5 of the 11 females the oogonia were cysted in groups of 4-8 cells surrounded by a thin fibrous layer. In the other 6 fish the oogonia appeared uncysted with some fibres positioned between them (ARF= 9.3%). Mitotic figures were frequent among the oogonia in all females examined.

A moderate proliferation of tubular structures very similar to renal tubules was evident interspersed between the oogonia in two of the triploid females studied (IRFs= 7.8 and 12.6%, respectively). Localised foci of necrosis (ARF= 5.8%) and a moderate infiltration of eosinophilic inflammatory cells (ARF= 1.9%) were evident under the germinal epithelium covering the outer surface of the lamellae.

4.4.2.2 *Gonadal structure at 15 months post-hatching.*

Most of the oocytes in the ovaries of diploid females at 15 months of age had reached stage 4, clearly identified by the presence of yolk vesicles staining pale mauve in their cytoplasm (ARF= 41.7%, Figure 4.12). Radial striations of the zona radiata were visible between the granulosa and the oocyte membrane. The granulosa cells appeared cubical in shape while thecal cells showed a flattened appearance. Large numbers of stage 3 oocytes were still evident (ARF= 32.7%), together with scattered oocytes at stage 2 (ARF= 5.2%). A few atretic follicles

(ARF= 2.4%) were also present, recognisable by a hypertrophied granulosa penetrating the zona radiata and a shrinking of the oocyte which had separated from the theca. The stroma around the follicles contained thin fibres and few erythrocytes (ARF= 7.4%).

Triploid females exhibited a closed lamellar ovarian architecture at this age. The gonad appeared delimited by a thick, fibrous tunica albuginea in which focal areas of cytolysis and necrosis were common (ARF= 5.6%). Fibres underneath the epithelium of the tunica infiltrated the underlying germinal tissue, which appeared replaced by adipose deposits in some areas. The columnar cells of the germinal epithelium appeared significantly enlarged (ARF= 3.9%, Figure 4.13) and some cell debris seemed to be washing up into the ovarian lumen. Within the lamellae, oogonia (ARF= 57.3%) appeared nested in groups of 4-20 cells surrounded by a thin fibrous layer in all 9 females examined (Figure 4.15). The oogonia had a stranded cytoplasm and very variable nuclear sizes with frequent signs of vacuolisation and karyorhexis, and occasional mitotic figures were observed. These cysts were surrounded by abundant pink-staining fibrous elements (ARF= 18.4%) with a central, elongated nuclei, and moderate amounts of inflammatory cells (ARF= 1.4%). Some dark-staining flattened cells with ellipsoidal nuclei reminiscent of thecal cells were also evident between the cysts. In addition, tubular structures resembling kidney tubules and abortive glomerulae were interspersed among the gonial cysts in three of the females examined (IRFs= 2.3-7.8%).

The ovary of three of the triploid females examined seemed to be undergoing a significant change: isolated foci of dark-staining areas (IRF= 0.8-6.8%) appeared to be developing among the gonial cysts, giving these zones a patchy appearance. Cells within these areas had a much smaller size than oogonia, and appeared morphologically very similar to primary spermatocytes (thereafter referred to as *spermatogenic cells*). These areas were not clearly delimited by an outer layer, but instead they were located in close proximity to the gonial cysts.

4.4.2.3 Gonadal structure at 20 months post-hatching.

All six diploid females examined were approaching ovulation at 20 months of age. The lamellar organisation of the ovary appeared masked by the large size of the oocytes which had completed growth to the late stage 6 of oogenesis (ARF= 50.2%, Figure 4.12). These oocytes were characterised by the presence of many large yolk globules in their ooplasm, the cortical alveoli being restricted to the area immediately adjacent to the oocyte membrane (Figure 4.16). Their nuclei or germinal vesicle presented a folded nuclear envelope and was slightly displaced from the centre. The zona radiata appeared thicker than in the previous sample, and the granulosa cells had also become more flattened.

Some oocytes were still at stage 5 (ARF= 9.2%) showing smaller yolk granules in the ooplasm adjacent to the oocyte membrane and a centrally positioned nucleus. A few atretic follicles were also evident in all females (ARF= 5.4%). These were characterised by a hypertrophied granulosa with an irregular basement membrane and appeared filled with acidophilic fragments of the zona radiata and yolk granules (Figure 4.16).

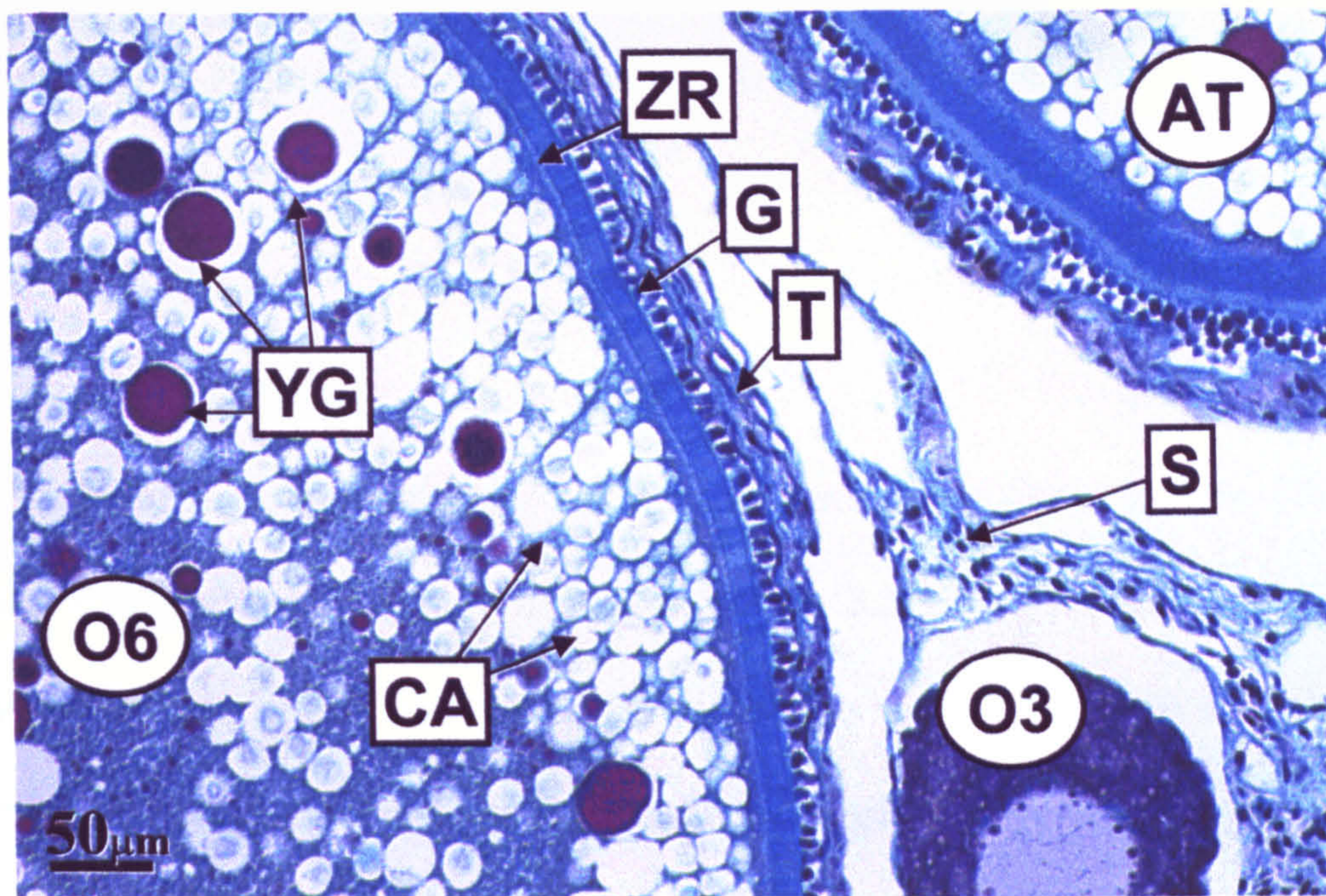


Figure 4.16. Gonadal photomicrograph from a 20 month-old diploid rainbow trout female stained with Masson's trichrome. AT, atretic oocyte; CA, cortical alveoli; G, granulosa; O3, stage 3 oocyte; O6, stage 6 oocyte; S, stroma; T, theca; YG, yolk granules; ZR, zona radiata.



Figure 4.17. Gonadal photomicrograph from a 20 month-old triploid rainbow trout female stained with haematoxylin and eosin. O, oogonia; O4, stage 4 oocyte; N, necrosis; TA, tunica albuginea; VL, vascular lacunae.

Low numbers of stage 3 (ARF= 4.2%) and 4 (ARF= 0.7%) oocytes were embedded in a well vascularised stroma.

The ovaries of the triploid females sampled at this age retained the general morphology observed in the previous sample (Figure 4.17). The gonad presented a closed lamellar architecture with a heavy infiltration of fibres (ARF= 13.7%), fat deposits and scattered foci of necrosis (ARF= 3.2%, Figure 4). Large *vascular lacunae* expanding under the germinal epithelium were evident in 3 of the 6 fish examined. These appeared as distended irregular spaces delimited by a discontinuous endothelium and filled with myeloid-resembling cells (IRFs= 2.2-5.6%) and some cell debris. Oogonia (ARF= 61.4%, Figure 3) appeared nested within the lamellae, surrounded by a fibrous connective layer and exhibiting frequent pyknosis and karyorhexis. The ovary of a single female presented a few normal follicles (IRF= 8.9%) with oocytes at stage 4 and a normally developed granulosa surrounded by flattened thecal cells. In two of the females sampled the patchy configuration previously described was clearly evident (Figures 4.18 and 4.19). Interspersed among the oogonia, spermatogenic cells showed dense chromatin staining and appeared similar in size and morphology to secondary spermatocytes (IRFs= 3.3% and 6.7%, respectively). No tubular formations were found at this age in any of the triploid females examined.

4.4.2.4 Gonadal structure at 25 months post-hatching.

All of the 10 diploid females sampled at 25 months of age had ovulated. Large numbers of post-ovulatory follicles occupied most of the gonad (ARF= 34.6%, Figure 4.12).

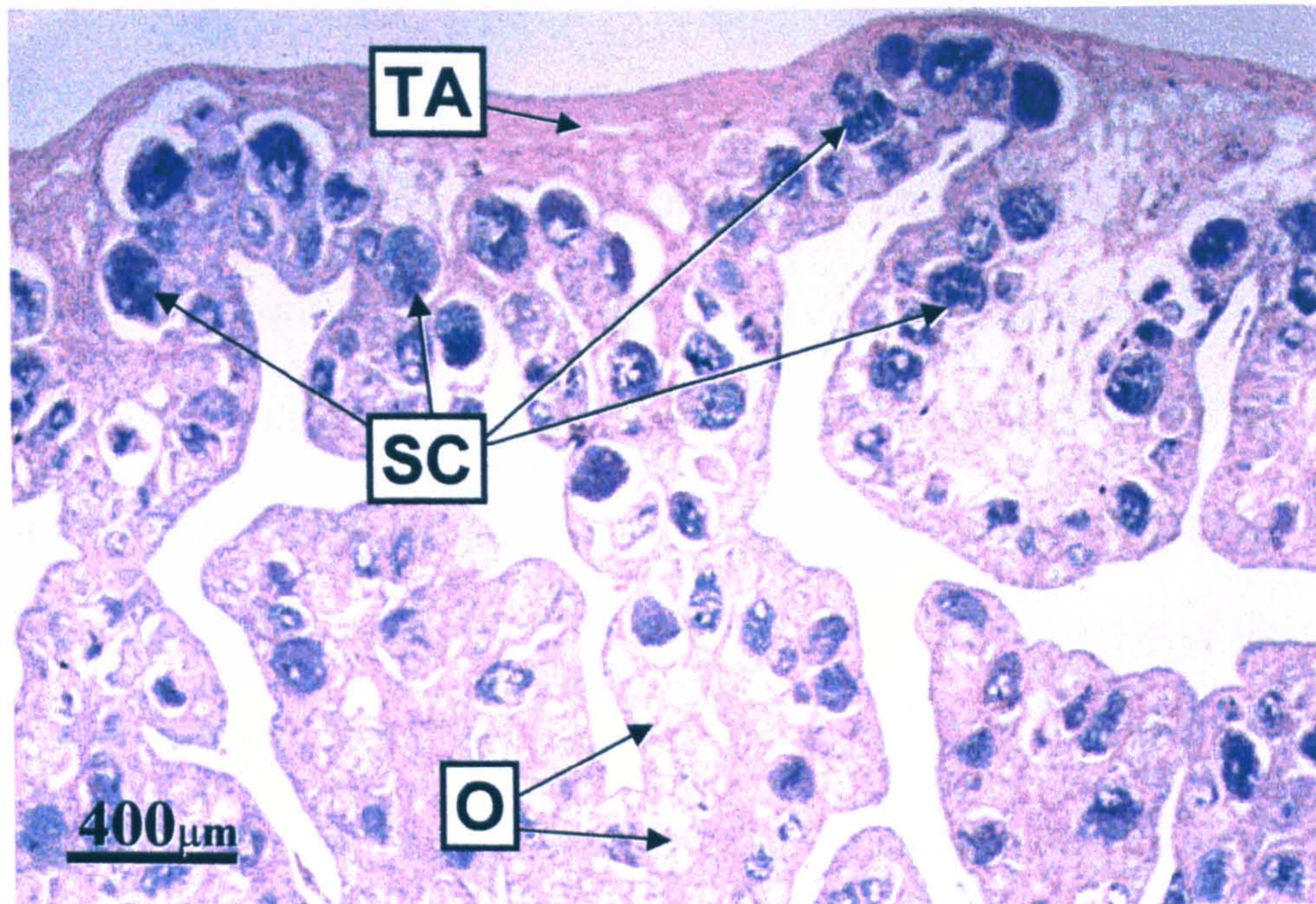


Figure 4.18. Gonadal photomicrograph from a 20 month-old triploid rainbow trout female stained with haematoxylin-eosin. O, oogonia; SC, spermatogenic cells; TA, tunica albuginea.

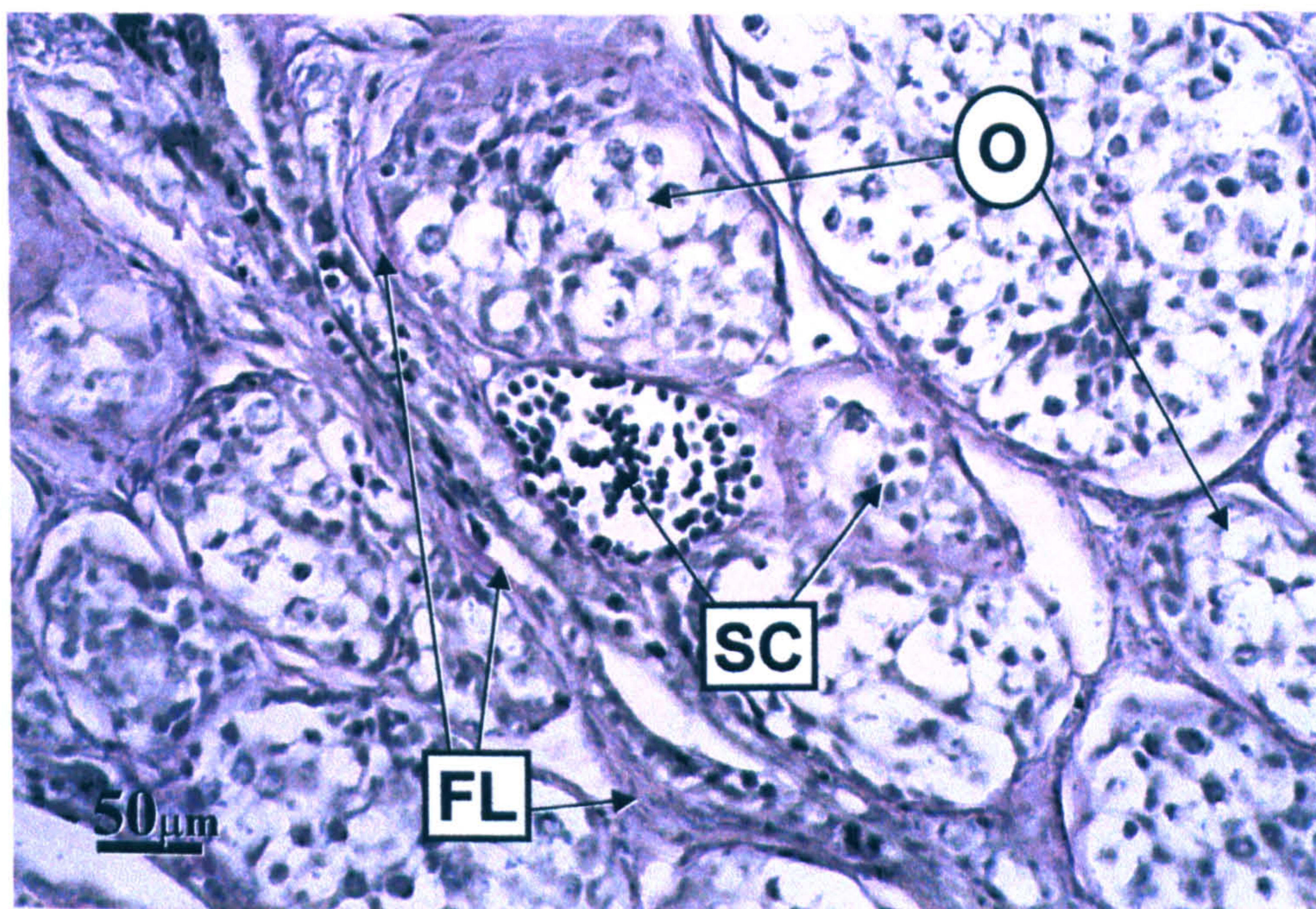


Figure 4.19. Gonadal photomicrograph from a 20 month-old triploid rainbow trout female stained with the periodic acid-Schiff reaction and counterstained with Heidenhain's haematoxylin. FL, fibrous layer; O, oogonia; SC, spermatogenic cells.

Post-ovulatory follicles appeared as collapsed structures with a proliferating granulosa filling the space previously occupied by the oocyte, and a theca which was continuous with the ciliated germinal epithelium. Variable numbers of atretic follicles (ARF= 9.2%) were also found in all ovaries examined. Atretic oocytes were separated from a hypertrophied granulosa, with the striations of the zona radiata still visible. The globular organisation of the yolk had been lost and instead a homogenous mass of acidophilic material filled the entire ooplasm. Post-ovulatory follicles and atretic oocytes appeared embedded in a loosely-bound stroma in which oocytes at stages 3, 4 and 5 were developing.

Although the general organisation noticed in the ovaries of triploid females during previous samples was conserved at 25 months of age, six of the ten females examined exhibited clear gonadal hermaphroditism at this age. Large areas of the ovary were occupied by small, darkly-staining cells morphologically identical to spermatocytes and spermatids, with no clear delimiting membrane (IRFs= 4.4-15.8%). Spermatogenic cells within these areas appeared to be undergoing meiosis, with spermatid-resembling cells centrally located and secondary spermatocytes in a more peripheral position.

Macroscopically, the gonad appeared as a fibrotic organ retaining a closed lamellar architecture in which spermatogenic areas and nests of 15-30 vacuolised oogonia (ARF= 43.4%) concurred with *vascular lacunae* (ARF= 1.5%), necrotic foci (ARF= 3.2%) and fat deposits. The lamellae appeared lined by a significantly enlarged germinal epithelium. In three of the ten females analysed low numbers of stage 4 oocytes (IRFs= 1.1-10.2%) appeared to be normally developing among the oogonial nests, frequently in close juxtaposition with spermatogenic areas.

These oocytes contained cortical alveoli in their cytoplasm, and had a clearly differentiated zona radiata, granulosa and thecal layer (Figure 4.20). The tubular formations observed in previous samples had invaded large parts of the ovary in three cases (IRFs= 14.4-24.4%, Figure 4.21). Cylindrical tubules contained a central lumen filled with an amorphous eosinophilic substance. The lumen was lined by a single layer of cubical epithelium with spherical nuclei centrally positioned.

4.4.2.5 Gonadal structure at 44 months post-hatching.

Diploid females were approaching a second ovulation and spawning at 44 months of age (Figure 4.12). The composition of the ovary appeared basically identical to that observed in the sample taken 25 months after fertilisation, with many oocytes at stage 6 (ARF= 28.9%) and a new wave of stage 3-4 oocytes developing within the stroma. Atretic follicles were numerous in all diploid females examined (ARF= 31.4%).

The lamellar architecture had been replaced by a more lobular appearance reminiscent of the testicular anatomy in six of the ten triploid females sampled at this age (Figure 4.22). Spermatogenic areas were now present in nine of the ten females examined (ARF= 12.2%), in most cases concomitant with oogonial nests (ARF= 30.4%). Many of the male-differentiating areas appeared to be at late stages of spermiogenesis (Figure 4.23), with most cells resembling spermatids and, at least in one case, morphologically abnormal spermatozoa in the central areas of some cysts (IRF= 38.6%).

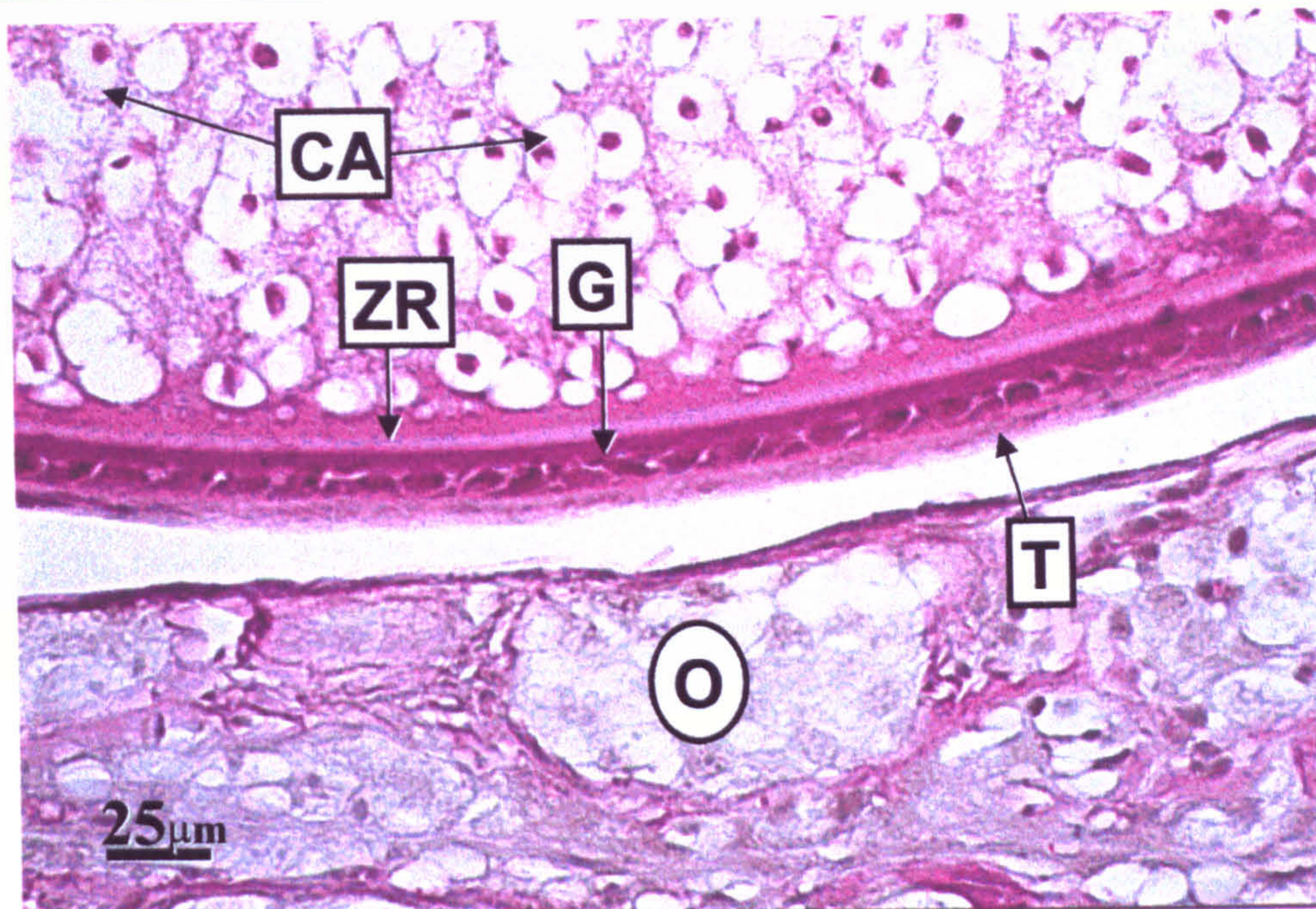


Figure 4.20. Gonadal photomicrograph from a 25 month-old triploid rainbow trout female stained with the periodic acid-Schiff reaction and counterstained with Heidenhain's haematoxylin. CA, cortical alveoli; G, granulosa; O, oogonia; T, theca; ZR, zona radiata.

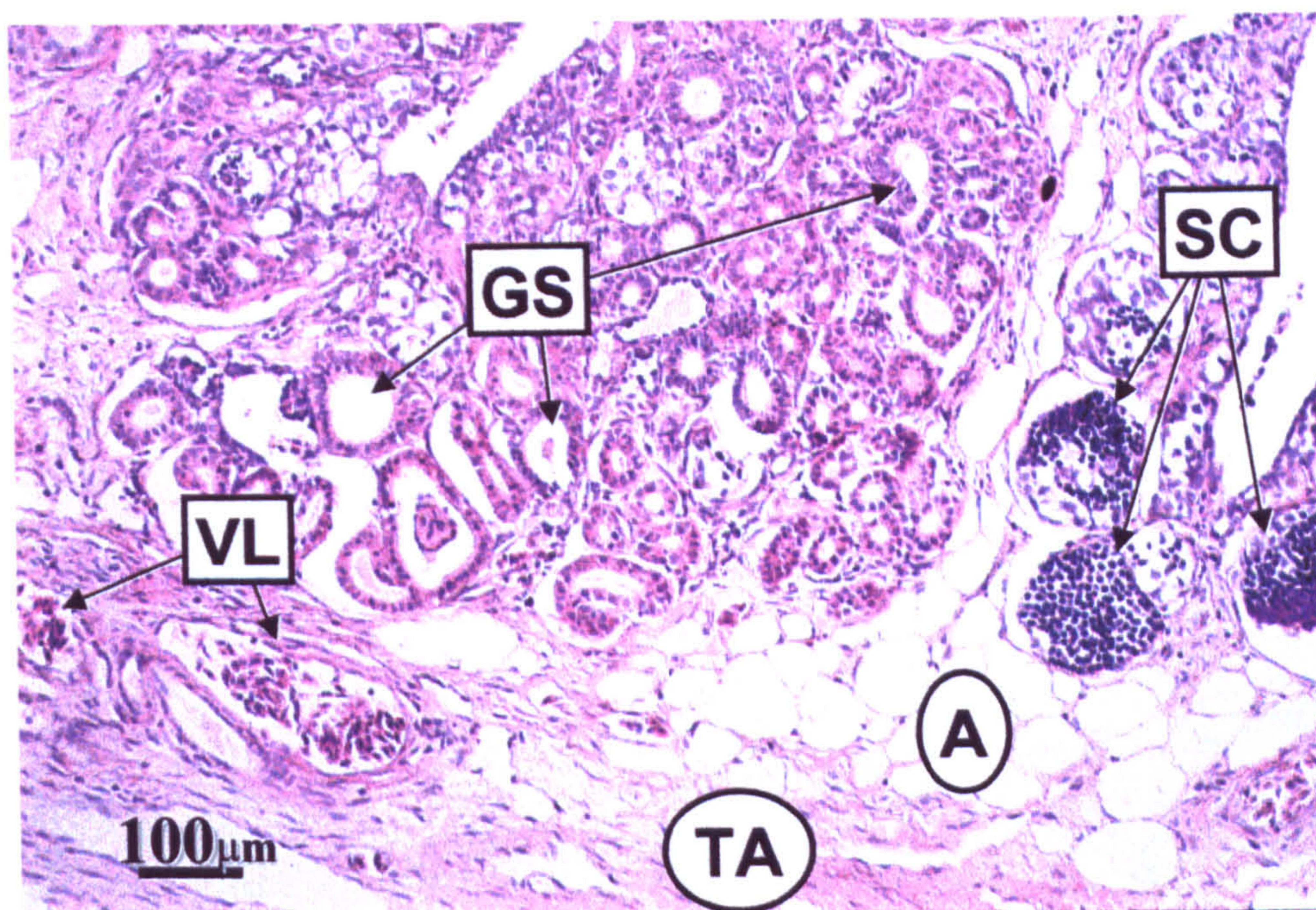


Figure 4.21. Gonadal photomicrograph from a 25 month-old triploid rainbow trout female stained with haematoxylin and eosin. A, adipocytes; GS, glandular structures; SC, spermatogenic cells; TA, tunica albuginea; VL, vascular lacunae.

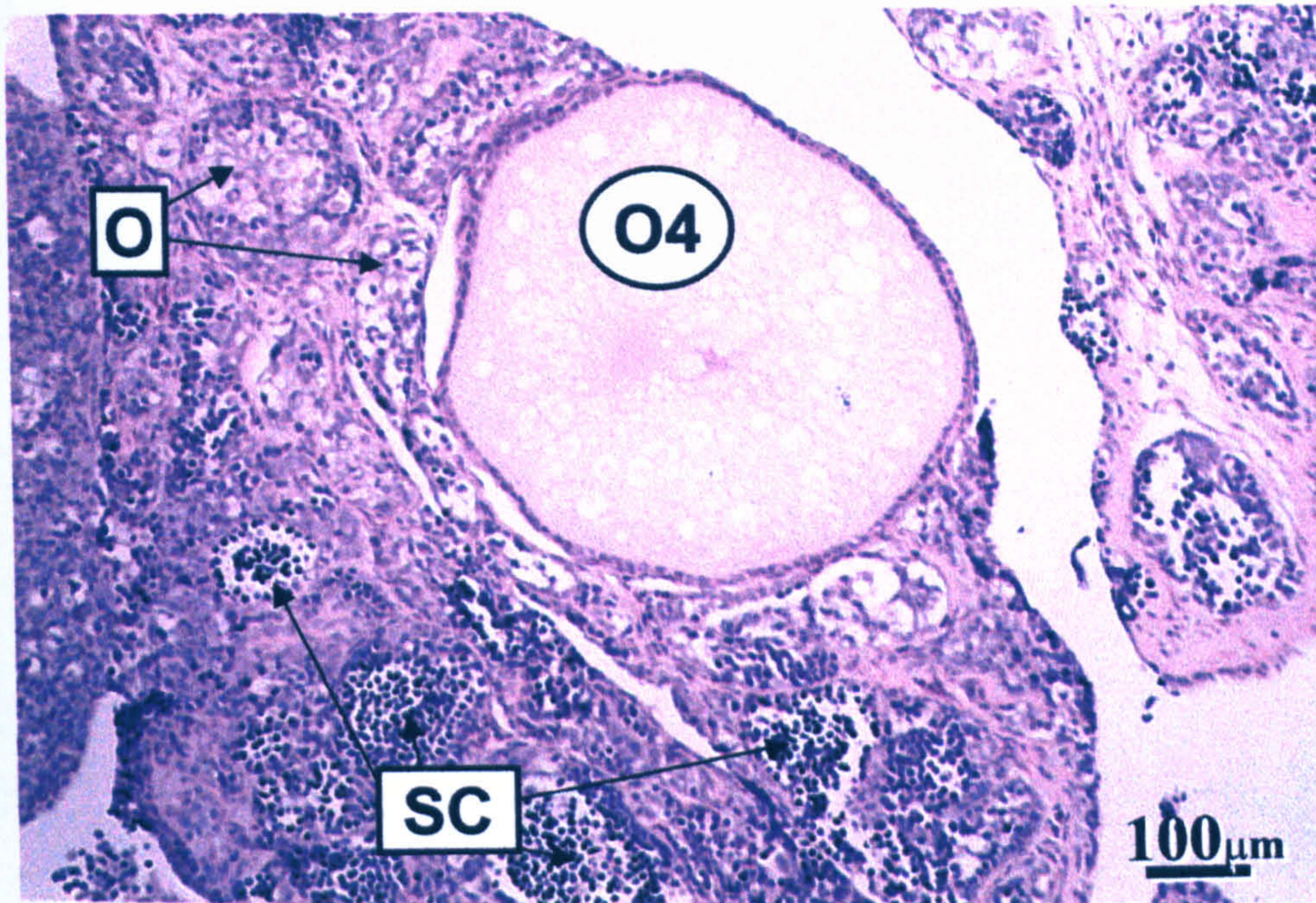


Figure 4.22. Gonadal photomicrograph from a 44 month-old triploid rainbow trout female stained with haematoxylin and eosin. O, oogonia; O4, stage 4 oocyte; SC, spermatogenic cells.

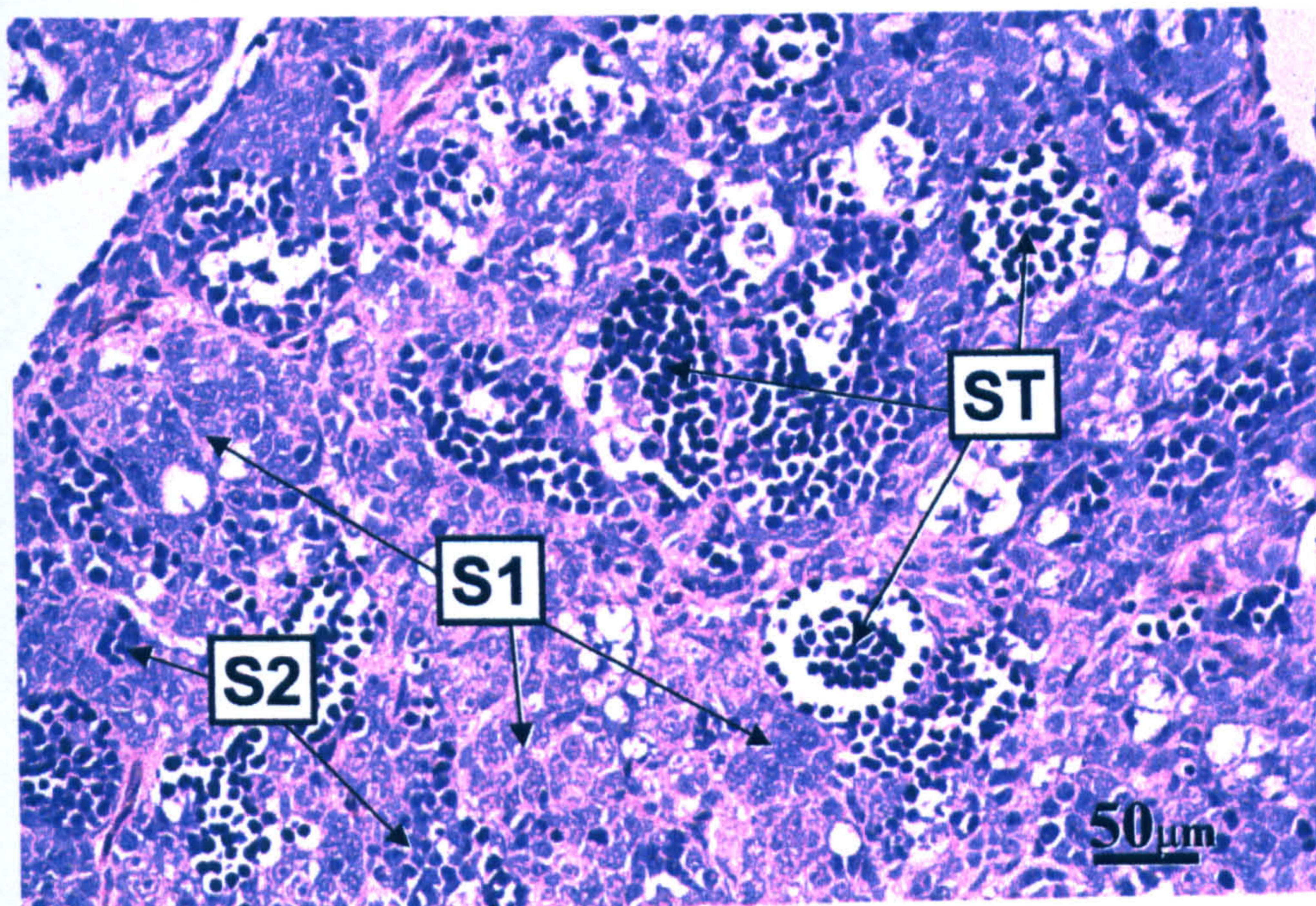


Figure 4.23. Gonadal photomicrograph from a 44 month-old triploid rainbow trout female stained with haematoxylin and eosin. S1, primary spermatocytes; S2, secondary spermatocytes; ST, spermatids.

Pyknosis and karyorhexis were frequent within the oogonial nests. A few normally developed follicles contained oocytes at stage 4 (ARF= 3.1%), and in three of the females these had entered stage 5 (IRFs= 3-9.5%) showing small exogenous yolk granules in the periphery of the ooplasm. Fibres were abundant (ARF= 16.8%) throughout the gonads of all triploid females, in some cases forming localised dense agglomerations of neoplastic appearance. Cylindrical structures similar to kidney tubules were evident in two females (IRFs= 3.3% and 33.3%, respectively). Fat deposits, *vascular lacunae* and necrotic foci were observed in all triploid females examined (Figure 4.13).

4.5 Discussion

4.5.1 Gametogenesis in triploid males

In general, the patterns of testicular development observed in triploid males in this study are in accordance with previous findings (Lincoln and Scott, 1984; Nakamura *et al.*, 1987; Kobayashi *et al.*, 1993). Triploid males progressed through initial phases of spermatogenesis (i.e. 7 and 15 months post-hatching) at similar or even faster rates than their diploid counterparts, and both ploidy groups contained precocious males that produced spermatozoa by the age of 15 months post-hatching. Differences were more apparent at the completion of spermatogenesis (20-25 months post-hatching), when all diploid males contained free spermatozoa in the lumen of most tubules, whereas triploid males contained predominantly spermatocytes and abnormal spermatids. Triploidy in rainbow trout males thus seems to have affected spermiogenesis (transformation of spermatids into spermatozoa) and spermiation rather than the proliferative (mitotic) or meiotic phases of spermatogenesis.

Spermiogenesis in the rainbow trout has been characterised as a complex differentiation process (Billard, 1983). Abnormal spermatid development has been reported in triploid fowls (Lin *et al.*, 1995b). Thus it is possible that in spite of mechanistic difficulties for chromosomal disjunction, progression through meiosis could be accomplished in a triploid cell, while the unbalanced gene expression that presumably unfolds in the aneuploid spermatids present in triploid males is insufficient in most cases to satisfactorily accomplish the differentiation into spermatozoa. Nevertheless, a few triploid individuals in the present study (one precocious male and 3 out of the 10 males sampled at 25 months of age) produced

morphologically normal spermatozoa. The appearance of normally differentiated spermatozoa may arise by chance from the random segregation of complete (haploid) chromosome sets. Alternatively, several cytological mechanisms capable of altering the chromosomal complement of triploid cells such as multipolar mitosis, acytokinetic mitosis, cell fusion or chromosome elimination may be operating in these males, as described in other polyploids (Pera, 1975; Nakai *et al.*, 1991, Ohtani, 1993). At present, the reasons for the individual variation detected in the effects of triploidy on testicular development remain unclear.

Early stages (7 months post-hatching) of spermatogenesis in triploid fish were also characterised by the presence of prominent Sertoli cells and a significant deficiency in interstitial (Leydig) cells (Figure 1). Sertoli cells are known to have phagocytic activity in teleosts (Billard, 1986). A possible explanation for the enlargement of Sertoli cells might relate to the slight necrosis (ARF= 0.69%) detected in triploid males, which was not observed in the diploid group at this age. Although the functional role of interstitial (Leydig) cells in teleosts is still subject to some discussion, they are thought to participate in steroid biosynthesis, particularly of 11-ketotestosterone (de Vlaming, 1974; Grier, 1981, Nagahama, 1994). The observed scarcity of these cells in triploid males might then explain the initially low levels of 11-ketotestosterone detected in maturing triploid trout males (Kobayashi *et al.*, 1993).

4.5.2 Gametogenesis in triploid females

Triploidy clearly affects ovarian development at an early stage, although in this study the absence of sampling before 7 months of age prevented a clear identification of the time of impact. The main features noticed in the ovarian development of triploid female trout were the persistence of the oogonial nests, the gradual appearance of spermatogenic areas and the extreme proliferation of non-follicular elements in all females examined.

Most germ cells remained enclosed in the oogonial cysts throughout the 44 months covered in the sampling period, with only a few oocytes developing outside the cysts during the last sampling. In the rainbow trout, as in other (but not all) salmonids and most vertebrate species, the germ cells are organised in clusters connected by intercellular bridges and surrounded by stromal cells (Takashima *et al.*, 1980; Van der Hurk and Slof, 1981; Nakamura and Nagahama, 1993). This organisation arises during the mitotic proliferation of oogonia, in which cytokinesis is frequently incomplete. The germ cells in each cluster undergo synchronous meiotic progression up to the late pachytene stage, and it has been suggested that the intercellular bridges within the gonial clusters play an important role in synchronising oocyte differentiation (Tokarz, 1978; Beers and Dekel, 1981). During early diplotene, the oocytes forming the clusters segregate and are gradually surrounded by somatic prefollicular cells (Peters, 1978). Primordial follicles formed in this way continue differentiation independently and asynchronously. The mechanisms leading to the disruption of the inter-oocyte bridges are still unclear, although it has been suggested that the thin cytoplasmic

digitations that stromal cells develop around the clustered oocytes could play an active mechanical role in this process (Andreuccetti *et al.*, 1990).

The presence of marginal numbers of oocytes developing outside the cysts to stages 4-5 in the present study would suggest that, rather than being due to the inability of the germ cells to progress through meiosis, the early blockage observed in triploid ovaries may have resulted from the sequestering of the oocytes within the gonial cysts. Furthermore, the observation of a normally-developed granulosa around these extra-cystic oocytes suggests that, provided that the proper interactions with the developing oocytes can be established, granulosa cells in triploid females retain the potentiality to differentiate correctly. Previous studies in salmonids have shown that the presence of a triploid genome results in an increase in cellular volumes (Small and Benfey, 1987). Perhaps the alteration in cellular dimensions interferes with the intimate cell-to-cell communication required for the disruption of the oogonial clusters in triploid females, resulting in the persistence of the oogonial nests. Further studies on the ultrastructure of the granulosa and oocytes in diploid and triploid female teleosts will be of great value for the clarification of the initial stages of the process of folliculogenesis.

An alternative hypothesis would consider that the lack of a proper hormonal environment prevents the disruption of the gonial cysts by inhibiting somatic-germ cell interactions, as it has been hypothesised during sex reversal in *Xenopus* (Villalpando and Merchant-Larios, 1990). Piferrer *et al.* (1994) detected no significant oocyte development in oestrogen-treated triploid females of coho salmon. However, extrapolation of information obtained in this species to the

rainbow trout may not be valid, since oogonia were not found to be naturally arranged in the cysts characteristic of normal ovarian development in rainbow trout, and the reproductive cycle of the coho salmon consists of a single spawn while the rainbow trout is a multiple spawner.

More recently, oestradiol and testosterone implants significantly increased sGnRH contents in the brain and pituitary of adult triploid rainbow trout (Breton and Sambroni, 1996). Krisfalusi and Cloud (1996) observed no differences in the ovarian development of triploid rainbow trout treated with 17β -oestradiol by immersion of eyed eggs and alevins and oral administration, compared to untreated triploids. Their findings propound that the supplementation of exogenous steroids during early ontogeny is insufficient to overcome the blockage in oocyte development observed in triploid female trout, suggesting that the significantly lower levels of gonadal steroids observed in female triploids (Lincoln and Scott, 1984; Nakamura *et al.*, 1987) are a result of reduced ovarian development, rather than the cause. The supplementation of exogenous 17β -oestradiol, however, may not be sufficient to restore the normal endocrine balance necessary for the initiation of folliculogenesis, which is likely to require both the establishment of the proper cellular interactions and the hormonal milieu necessary for the differentiation of the prefollicular cells and oogonia (Tokarz, 1978; Andreuccetti *et al.*, 1990; Villalpando and Merchant-Larios, 1990; Moley and Schreiber, 1995).

An important feature of ovarian development in the triploid females in this study was the gradual increase in frequency of male-differentiating areas observed between the ages of 15 months (3 of the 9 females examined, IRFs= 0.8-6.8%) and

44 months post-hatching (9 of the 10 females examined, IRFs= 1.8-38.6%). Interestingly, the ovarian development of chromosomally female (3A:ZZW) triploid chickens follows a similar pattern. Most left gonads of ZZW triploid chickens appear as normal ovaries at hatching, but subsequently the gonad becomes an ovotestes as testicular tissue develops to produce abnormal spermatozoa by 25 weeks of age (Thorne *et al.*, 1988; Fitzgerald and Cardona, 1993; Lin *et al.*, 1995a). Testicular differentiation also occurs in mouse foetal ovaries after elimination of germ cells (Hashimoto *et al.*, 1990). In the mouse, male somatic cells are autonomously committed to differentiate into Sertoli and peritubular (Leydig) cells, while specific somatic-germ cells interactions are required for the normal differentiation of female somatic cells (Burgoyne *et al.*, 1988). In the absence of such interactions, female somatic cells differentiate into testis cords containing Sertoli and peritubular cells, but no other testis-specific cells (Hashimoto *et al.*, 1990).

Trout and chicken germ cells retain the potentiality to differentiate in either male or female directions, as shown by the readiness with which sex reversal can be induced in these species (Bye and Lincoln, 1986; Wartenberg *et al.*, 1992). Thus it seems possible that in triploid trout females, the persistence of the oogonial cysts precludes the establishment of the somatic-germ cells interactions necessary for female somatic cell differentiation. Female somatic cells may then differentiate into testis-supportive cells (Sertoli and Leydig cells), which could in turn favour the masculine differentiation of pluripotential germ cells in the rainbow trout as well as in the chicken. In the absence of further information (i.e. ultrastructural studies

on the ovarian development of triploid rainbow trout) this hypothesis remains speculative.

Finally, the extreme proliferation of non-follicular components observed in triploid females is difficult to interpret, not only because of the diversity of the structures observed (i.e. *vascular lacunae*, fibrous conglomerates, tubular proliferation) but also in view of the current scarcity of information on their origin and function in the vertebrate ovary. For instance, the significant enlargement of the germinal epithelium detected in triploid females in three of the samples performed is difficult to explain, since although it seemed to be closely associated with the development of satellite or follicle cells for adjacent germ cells, the functional role of the germinal epithelium remains unclear (Duke, 1978). Wilcox and Mossman (1945) claimed that testis-like cords observed in the ovaries of *Sorex vagrans*, the vagrant shrew, originated in the germinal epithelium. Price (1953) described the thickening of the germinal epithelium in the anoestrous water shrew, although no significance was attributed to this seasonal activity.

Tumour-like cell masses resembling tubular adenomas have been described in the ovarian development of triploid (3A: ZZW) intersex chickens (Frankenhuis, 1988) as well as in mouse ovaries deficient in germ cells (Duncan and Chada, 1993). The presence of extensive myeloid centres has been recorded in the ovary of elasmobranchs, in which haematopoietic elements from the kidney replace the original medullary tissue and persist there (Matthews, 1950; Franchi, 1962). Leukocytes infiltrate the gonads of triploid ZZW fowl (Lin *et al.*, 1995a). The observation of inflammatory cells, fibrosis and necrosis would suggest the presence of some type of immune response during the ovarian development of

triploid females, which may be related to the appearance of the proliferative structures mentioned before.

Overall, it seems that non-follicular components undergo abnormal differentiation reflecting a certain degree of pluripotentiality in the triploid females examined. For example, the glandular formations resembling kidney tubules may represent overgrown remnants of the *rete ovarii*, which originates in the tubular connection that develops between the mesonephros and the gonad during organogenesis. The *rete ovarii*, however, is normally absent in teleosts, first appearing in the ovaries of vertebrates in the Amphibia (Duke, 1978). Although in mammals it has been suggested that the *rete* system interacts with the ovarian cortex initiating the start of meiosis (Byskov, 1975), its ontogeny and functional role remain obscure.

CHAPTER V

THE REPRODUCTIVE POTENTIAL OF RAINBOW TROUT TRIPLOID MALES, *ONCORHYNCHUS MYKISS*

5.1 Summary

In order to evaluate the reproductive potential of rainbow trout autotriploid males, six adult triploid males were selected from an experimental population for the analysis of their gonadal parameters and breeding capacity. Testicular weight, gonado-somatic index, sperm cell density and spermatozoa motility were significantly lower in triploid than in diploid male siblings. Ova collected from diploid female trout were inseminated with sperm from autotriploid males, diploid males or with a mixture of both ($2n \times 3n$, $2n \times 2n$ and $2n \times [2n+3n]$ crosses). Survival from fertilisation to hatching was 0.57% in $2n \times 3n$ crosses, 10.7% in $2n \times 2n$ crosses and 10.9% in $2n \times [2n+3n]$ crosses, and it was affected by low egg quality. Survival from hatching to 4 months was 10%, 66% and 44% for the $2n \times 3n$, $2n \times 2n$ and $2n \times [2n+3n]$ crosses ($P < 0.05$). Hatching embryos in $2n \times 3n$ crosses exhibited morphological abnormalities, although some juveniles were obtained and one of them had developed large testes in meiotic phase.

Ploidy of juveniles was examined by image cytometry of modified Azure A-stained blood and liver smears. Image analysis of nuclear DNA in erythrocytes and hepatocytes revealed a near-triploid genome in $2n \times 3n$ offspring and in 12.5% of $2n \times [2n+3n]$ progeny. Metaphase plates analysed in gill epithelia from these individuals revealed aneuploid figures and multiple levels of ploidy. These data provide first evidence for the generation of limited numbers of viable progeny by autotriploid rainbow trout males when crossed to diploid females.

5.2 Introduction

Induction of triploidy by physical or chemical treatment of freshly fertilised ova has become a valuable tool in salmonid culture, enhancing growth at pubertal age, preserving flesh quality and minimising mortality during the spawning season (Purdom, 1983; Thorgaard, 1986; Ihssen *et al.*, 1990; Hussain, 1996). Triploids are believed to be sterile because of abnormal meiosis, although the degree of reproductive suppression depends on sex and species (Lincoln, 1981; Wolters *et al.*, 1982; Benfey and Sutterlin 1984a; Brämick *et al.*, 1995).

The gonadal development in triploids has been documented in salmonids (Benfey and Sutterlin, 1984; Lincoln and Scott, 1984) and non-salmonid species (Lincoln, 1981; Wolters *et al.*, 1982; Ueno, 1985). Gametogenesis and sexual maturation are arrested in triploid females, while triploid males display secondary sex characters and courtship behaviour (Kitamura *et al.*, 1991), develop an endocrine profile similar to diploid males (Benfey *et al.*, 1989, Nakamura *et al.*, 1993), and undergo aneuploid spermatogenesis (Benfey *et al.*, 1986). In non-salmonid species such as plaice and grass carp, survival of the offspring from triploid males crossed to diploid females was found to be extremely low due to developmental abnormalities, abnormal hatching and high larval mortality (Lincoln and Scott, 1984; Van Eenennaam *et al.*, 1990). Ueda *et al.* (1991) describe the production of hypertriploid progeny in crosses between diploid rainbow trout females and allotriploid males (rainbow trout × brook trout, *Salvelinus fontinalis*). No direct experimental evidence on the generation of viable progeny by autotriploid males in rainbow trout has been provided to date.

As described in Chapter 4, during the first spawning season a number of males in the triploid group displayed secondary sex characters (dark coloration and changes in body shape), although they did not exhibit natural spermiation. This Chapter describes the assessment of the reproductive potential of these individuals and the characterisation of any potential offspring.

5.3 Materials and Methods

5.3.1 Experimental animals

Triploid rainbow trout were produced from crosses of Mt. Lassen-derived strains. Triploidy was induced by a 13-min, 27°C heat shock applied to eggs 11 min post-fertilisation. Triploid and diploid (siblings) were raised in separate tanks at similar density, feeding and water quality regimes (T= 10-18 °C, ambient photoperiod). Six triploid males (age= 20 months post-hatching, 1.85 kg mean body weight) and six diploid males (same age, 1.9 kg mean body weight) were injected intramuscularly with 25 mg/kg LHRH analogue (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) to induce spermiation. Forty-eight hours after injection, they were anaesthetised (tricaine methansulfonate, Sigma Chemical Co., St. Louis, Missouri, U.S.A.) for sperm collection, and later necropsied to obtain blood, liver samples and morphometric parameters. Ploidy was evaluated by nuclear size of red blood cells measured with a Coulter Counter and Channeliser (Johnson *et al.*, 1984). Sperm was shipped on ice in oxygenated plastic bags and analysed for cell density and spermatozoa motility in the laboratory of Dr. G. Thorgaard (Washington State University, Pullman, Washington, U.S.A.). Evaluation was conducted 48 hr after sperm collection.

Approximately 1 ml of sperm from each male was used to inseminate eggs stripped from diploid females. Random batches of 300 eggs collected from 3 diploid females were used for insemination. The following crosses were conducted: six $2n \times 3n$ crosses, six $2n \times 2n$ crosses and three $2n \times [2n+3n]$ crosses (1:1 volume mixture of sperm from triploid and diploid males). All precautions were taken to prevent the possibility of sperm cross-contamination. Ova were stripped into a dry bowl, sperm

was added by pipette and briefly mixed, and hatchery water was added with gentle stirring for 2 min. Fertilised ova were rinsed in hatchery water and stocked in indoor 15 L fibreglass tanks covered with dark plastic screens to reduce illumination ($T=13.6 \pm 0.8^{\circ}\text{C}$). Photoperiod was adjusted to natural light cycles, and unfertilised ova and dead embryos were removed and counted daily for the computation of survival rates. At 5 hr after insemination all tanks were sampled for fertilisation success by fixing ~100 eggs in Stockard's solution. Swim-up fry were fed a commercial diet *ad libitum*. At 4 months of age, all surviving progeny were sacrificed and blood and liver imprints prepared and fixed in 10% phosphate-buffered formalin.

5.3.2 Ploidy determination

DNA content (pg/cell) was examined on formalin-fixed smears by using a CAS-200 Image Analyser (Cell Analysis Systems, Elmhurst, Illinois, U.S.A.) fitted with Quantitative DNA Analysis[®] software and a 280 μm filter with a 20 nm bandpass (Teplitz *et al.*, 1990). Rat liver nuclei (DNA=6.6 pg/cell) were used as external standards. A total of 90 smears were analysed (45 blood smears, 45 liver smears), with a mean number of 116 nuclei examined per slide (erythrocytes and hepatocytes). After a 70 min acid hydrolysis (0.1 N HCl), smears (blood and liver) were dipped in modified Azure A stain for 60 min, washed in 0.05 N HCl and dehydrated in acid alcohol (1:100 solution of 37% HCl and 70% ethanol), 100% alcohol and xylene. Four separate quadrants of a smear were analysed in order to ensure random sampling. Cell types used for ploidy determination were verified on adjacent serial paraffin sections stained by haematoxylin-eosin.

5.3.3 Chromosomal analysis

Several individuals among the offspring were karyotyped. For the preparation of the chromosomes, fragments of the gill arches were dissected, cut in small pieces and incubated in phosphate buffered saline containing 25 µg/ml colchicine for 5 hr at 14°C. Tissue fragments were washed in cold sodium citrate and stored in 3:1 methanol: acetic acid. After fixation, the specimens were gently minced in 45% glacial acetic acid and dropped onto clean microscope slides heated to 40-50°C. Slides were stained with undiluted Giemsa, transferred to NH₄OH, rinsed in acetone, xylenes and mounted (Thorgaard and Disney, 1990). Metaphase plates and histological sections were photographed with an Olympus BH-2 microscope.

5.3.4 Statistical analysis

Student's *t*-test was used to compare morphometric parameters of broodfish. Proportions of surviving individuals from fertilisation to hatching and from hatching to 4 months were arcsine-transformed, and survival in all crosses was compared by a one-way analysis of variance using a random block design (block= individual females). Significant differences in DNA content were evaluated by a one-way analysis of variance for unbalanced data (GLM Procedure, SAS Institute Inc., Cary, North Carolina, U.S.A.), and Scheffe's confidence intervals with a 95% joined level of confidence constructed when appropriate (Neter *et al.*, 1990). The significance level reported for all observations was $P < 0.05$.

5.4 Results

5.4.1 Broodstock

Data on males morphometry and sperm quality are shown in Table 5.1. Weight of testes and gonado-somatic index were significantly lower in triploid males compared to diploid males. Sperm collected from triploid males had a very low cell density (including two males with no spermatozoa) and immotile spermatozoa at 48 hr after sample collection.

5.4.2 Survival of the Progeny

The failure of the fixation protocol for consistently preserving cellular structure in triploid and control groups made impossible the determination of fertilisation success at 5 hr post-fertilisation. Eyed embryos were noticed in all crosses at day 11 after insemination, and hatching took place 20-24 days after insemination. The survival of embryos from incubation to hatching was low and variable in all crosses including control ($2n \times 2n$) matings (Table 5.2). The analysis of variance revealed a significant female effect and no significant differences between the three crosses within a single egg source. Hatching success was $0.6\% \pm 1.4$, $10.7\% \pm 16.7$ and $10.9\% \pm 9.8$ for the $2n \times 3n$, $2n \times 2n$ and $2n \times [2n+3n]$ crosses, with only one cross in the $2n \times 3n$ group (triploid male number 2, Table 5.1) producing hatched fry (8 individuals of 235 eggs stocked, 3.4%). The majority of the embryos in the $2n \times 3n$ group exhibited abnormal morphology with bent notochords and tail deformities. They seemed to have difficulties at hatching, only partially exiting the egg shell.

MALE	BW (g)	FL (cm)	K	GW (g)	GSI (%)	LW (g)	HSI (%)	M (%)	SCD (cells/mm ³)
TRIPLOIDS									
1	2244	56	1.28	33.1	1.47	19.8	0.88	0	17.300
2	1869	53	1.26	37.2	1.99	20.8	1.11	0	27.050
3	1155	46	1.19	9.1	0.78	10.5	0.91	0	3.100
4	1921	54	1.19	12.8	0.66	22.0	1.15	0	ABSENT
5	1635	47	1.57	19.9	1.22	15.1	0.92	0	1.450
6	2312	56	1.33	49.3	2.13	20.1	0.87	0	ABSENT
MEAN	1856	52	1.30	<u>26.9</u>	<u>1.38</u>	18.1	0.97	<u>0</u>	<u>8.150</u>
SD	388	4	0.13	14.2	0.55	4.0	0.11		10.361
DIPLOIDS									
1	1901	53	1.28	61.8	3.25	19.6	1.03	n.a.	n.a.
2	2155	54	1.37	73.1	3.39	26.2	1.21	60	20.27x10 ⁶
3	1658	47	1.60	55.8	3.37	15.9	0.96	90	NORMAL
4	2218	55	1.33	52.4	2.36	15.9	0.72	80	19.78x10 ⁶
5	2056	53	1.34	70.3	3.42	17.4	0.85	90	NORMAL
6	1361	44	1.60	52.8	3.88	13.1	0.96	10	19.28x10 ⁶
MEAN	1892	51	1.42	<u>61.0</u>	<u>3.28</u>	18.0	0.95	<u>66</u>	<u>19.77x10⁶</u>
SD	300	4	0.13	8.2	0.46	4.1	0.15	30	0.40x10 ⁶

Table 5.1. Morphometric data and sperm quality in triploid and diploid rainbow trout males. Key: BW, body weight; FL, fork length; GSI, gonadosomatic index (gonadal weight/body weight)×100; GW, gonadal weight; HSI, hepato-somatic index (liver weight/body weight)×100; K, condition factor (body weight/fork length³)×100; LW, liver weight; M, sperm motility; n.a., not available; SCD, Sperm Cell Density; SD, standard deviation. Diploid males labelled NORMAL were not examined due to small volume of sample, but their cell density appeared to be similar to the diploids that were analysed. Underlined values denote significantly different means ($P<0.05$).

Dark pigmentation was first observed in $2n \times 2n$ and $2n \times [2n+3n]$ groups by day 8 after hatching and by day 16 in the $2n \times 3n$ group. Exogenous feeding started in $2n \times 2n$ and $2n \times [2n+3n]$ groups on day 15 post-hatching. The majority of the fry in the $2n \times 3n$ group remained on the bottom of the tank, although some individuals in this group appeared normal, actively swimming to the water surface and accepting food. Significant differences in survival between groups were observed at both swim-up and fingerling stages (Table 5.2).

STAGE	PERCENT SURVIVAL		
	$2n \times 2n$	$2n \times 3n$	$2n \times [2n+3n]$
fertilisation to 'eyed' (day 11)	46.5±35.5(n=6)	45.0±35.5(n=5)	50.6±43.3(n=3)
fertilisation to hatching (day 20)	10.7±16.7(n=2)	0.6±1.4(n=1)	10.9±9.8(n=2)
hatching to swim-up (day 35)	66.0±3.5 ^a (n=2)	30.0 ^c (n=1)	45.6±1.7 ^b (n=2)
hatching to 4 months (day 113)	65.6±3.0 ^a (n=2)	10.0 ^c (n=1)	44.4±2.9 ^b (n=2)

Table 5.2. Percent survival for the three types of crosses performed at different developmental stages. Values are means ± standard deviations. n= number of crosses examined at each stage. Different superscripts denote significantly different means ($P < 0.05$).

Overall survival from hatching to four months was 10%, $66\% \pm 3$ and $44\% \pm 3$ for the $2n \times 3n$, $2n \times 2n$ and $2n \times [2n+3n]$ groups. Out of a total of 15 crosses performed, one $2n \times 3n$, two $2n \times 2n$ and two $2n \times [2n+3n]$ crosses yielded viable individuals at 4 months of age. A single $2n \times 3n$ cross produced a 4 month-old juvenile (10.2 cm fork length, 14 g body weight, Figure 5.1, A). Histological analysis of the gonads of this individual revealed the presence of testes in meiotic phase of spermatogenesis (Figure 5.1, B).

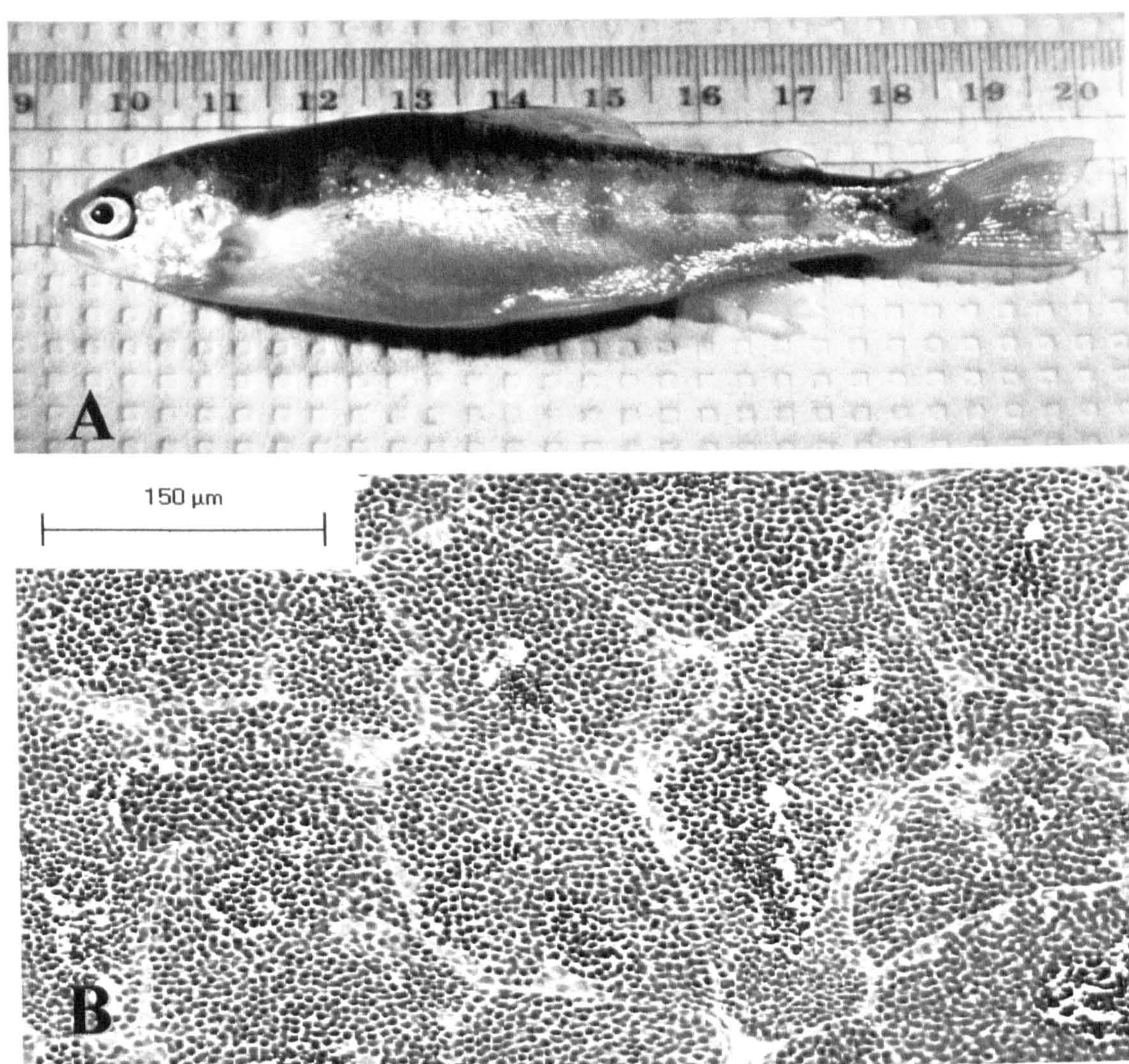


Figure 5.1.

A, 4 month-old maturing male from a $2n \times 3n$ cross. Scale of rule is in centimetres.

B, Microphotograph of a testicular section from the fish in Figure 5.1A showing meiotic phase of spermatogenesis (haematoxylin-eosin stain). Scale bar= 150 μm .

5.4.3 Image Cytometry of DNA

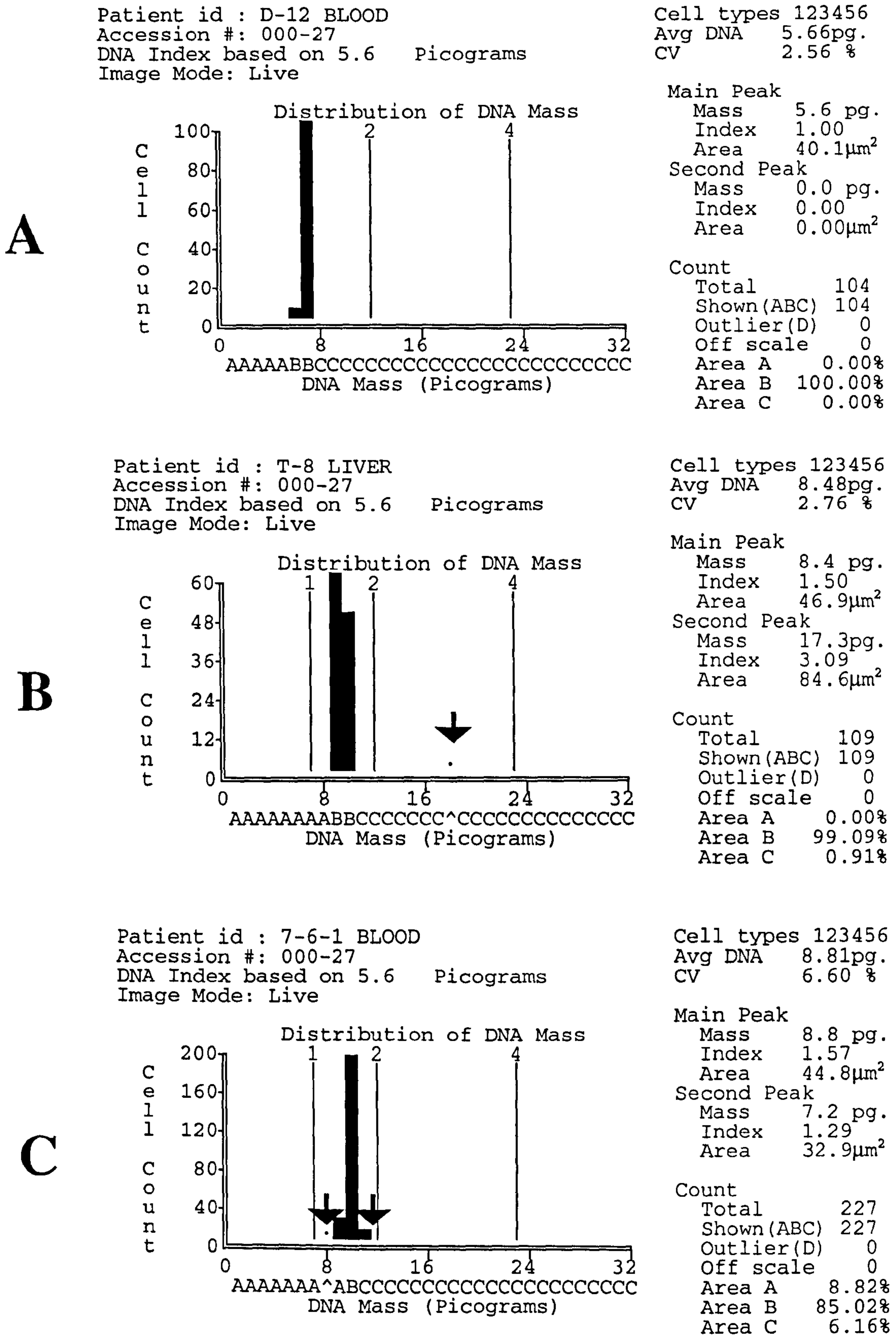
Average DNA values observed for parents and offspring on blood and liver smears are summarised in Table 5.3. Three different kinds of DNA distribution were found in blood and liver smears: a narrow distribution with a coefficient of variation under 5% and an average DNA value of 5.28-5.71 pg/cell was found in diploid broodfish, $2n \times 2n$ offspring and in 87.5% of the $2n \times [2n+3n]$ offspring (66 analyses performed in 32 fish, Figure 5.2, A). A similar distribution with a coefficient of variation under 5% but with an average value of 8.19-8.43 pg/cell of DNA was observed in triploid male parents (12 analyses performed in 6 fish, Figure 5.2, B). Finally, a wider histogram with a coefficient of variation over 5% and an average DNA value ranging from 8.19 to 8.83 pg of DNA per cell was recorded in $2n \times 3n$ offspring and in 12.5% of the $2n \times 2n+3n$ progeny studied (12 analyses in 3 fish, Figure 5.2, C).

GROUP	DNA (pg/cell)	
	BLOOD	LIVER
2n PARENTS	5.46 ± 0.21^a (n=9)	5.28 ± 0.16^a (n=9)
3n MALES	8.43 ± 0.19^b (n=6)	8.19 ± 0.40^b (n=6)
OFFSPRING		
$2n \times 2n$	5.57 ± 0.27^a (n=11)	5.64 ± 0.24^a (n=11)
$2n \times [2n+3n]$	5.44 ± 0.33^a (n=12)	5.71 ± 0.15^a (n=12)
	8.83 ± 0.47^c (n=2)	8.82 ± 0.20^c (n=2)
$2n \times 3n$	8.81 ± 0.70^c (n=1)	8.63 ± 0.62^c (n=1)

Table 5.3. Average DNA content observed on blood and liver Azure A-stained smears. n, number of fish. Data are means \pm standard deviations. Different superscripts denote significantly different means ($P < 0.05$).

Differences among these three types of DNA distribution were statistically significant ($P < 0.0001$), and multiple comparison of means by Scheffe's confidence intervals revealed three different ploidy levels: diploid, triploid and aneuploid/near-triploid.

Polyploid cells were detected in liver smears from all three ploidy groups (diploid, triploid and aneuploid/near-triploid), and in both parents and offspring. Polyploid hepatocytes were most abundant in diploid offspring (an average of 3.74% of the cells analysed per individual), followed by aneuploid/hypertriploid offspring (3.72%), diploid parents (1.65%) and triploid parents (0.57%). The average DNA values of the polyploid cells fell within the tetraploid range for the diploids (10.71 ± 0.38 pg/cell for the parents, 11.34 ± 0.56 pg/cell for the offspring) and the hexaploid range for the triploids/hypertriploids (17.05 ± 0.25 pg for the triploid parents, 16.87 ± 0.80 for the aneuploid/hypertriploid offspring). Polyploid cells were not found in blood smears.



5.4.4 Chromosomal analysis

Metaphase plates obtained from gill epithelia of the three aneuploid/near-triploid individuals revealed the presence of aneuploid figures with a variable number of chromosomes (mean fundamental number= 147 ± 22 , mean \pm s.d., $n=7$), but were in general similar to the triploid karyotype ($3n=90$, fundamental number=156). In addition, contrasting metaphase plates showing a grossly different number of chromosomes within the same individual were found in these three fish (Figure 5.3, A and B).

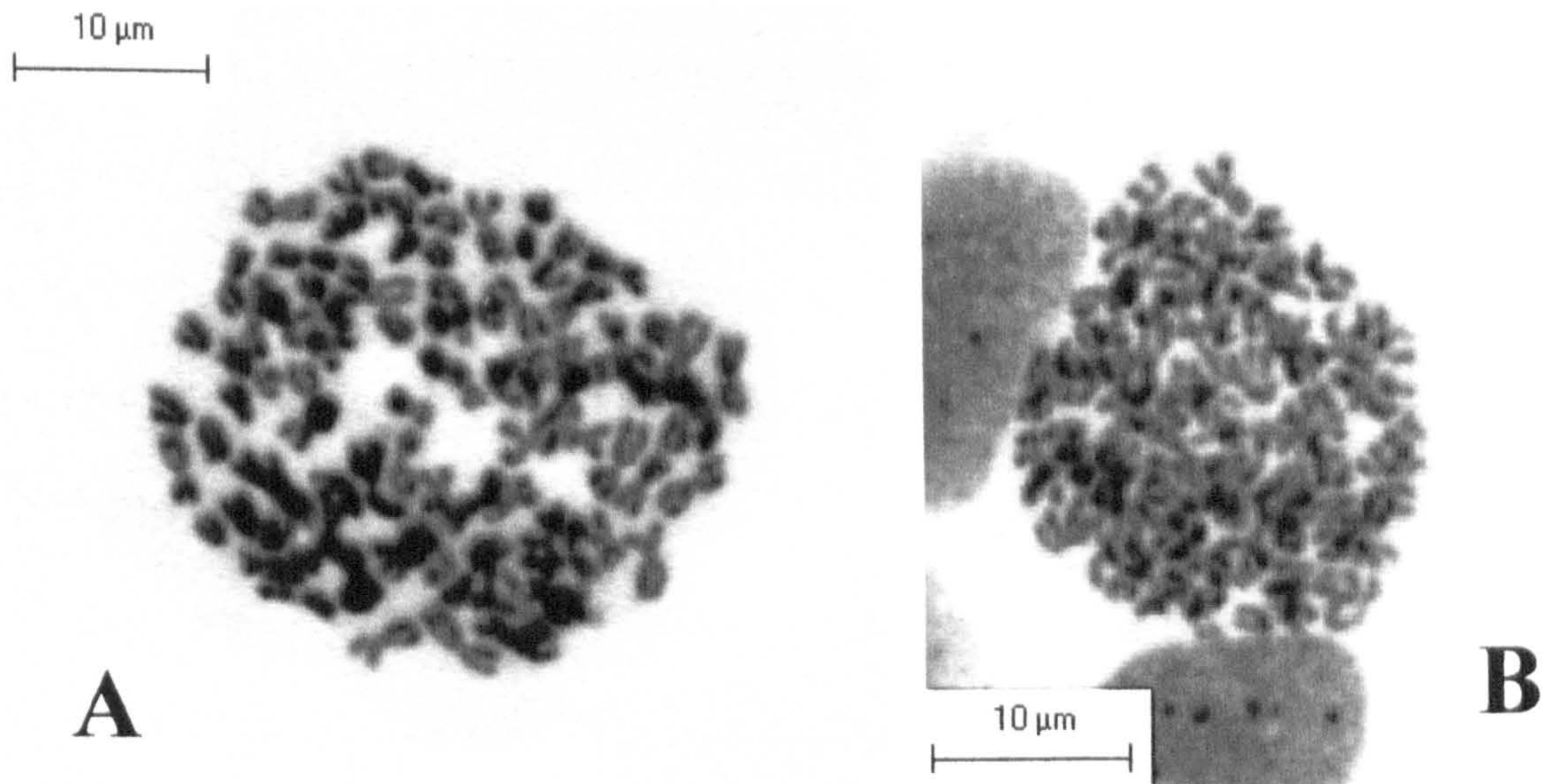


Figure 5.3.

A, Metaphase plate from gill epithelium of the fish in Figure 5.1 A. Scale bar= 10 μ m.

B, Metaphase plate from gill epithelium of a 4 month-old individual from a $2n \times [2n+3n]$ cross. Scale bar= 10 μ m.

5.5 Discussion

Triploid males used in this study displayed secondary sex characters and 4 of 6 were able to produce low-density sperm, although they had a significantly lower gonado-somatic index than diploid males. The motility of spermatozoa was not examined in freshly collected sperm, but the lack of sperm motility in triploid males 48 hr after sperm collection would not have precluded fertilisation capability. The significant motility detected in diploid spermatozoa after the same storage time indicated a higher viability and quality of the normal spermatozoa.

The survival of embryos reported in this work was unusually low for trout in all the crosses performed. The high mortality observed in all matings from the beginning of the incubation period was most likely associated with poor egg quality resulting from high water temperature (18°C) in the broodstock rearing tanks at the end of vitellogenesis, as well as the elevated temperature in the experimental tanks throughout the incubation period. In spite of these technical limitations, this study provides clear evidence for the occasional generation of limited numbers of progeny by autotriploid rainbow trout males.

The results obtained by image analysis in the present study were in full agreement with our findings obtained by flow cytometry of erythrocytes (Pérez-Carrasco *et al.*, 1994). Aneuploid/near-triploid progeny were obtained in one $2n \times 3n$ cross (triploid male number 2) and was also detected in two $2n \times [2n+3n]$ crosses (triploid males numbers 1 and 2). Both triploid males had the highest sperm densities among the males used in this study, although their GSIs and sperm densities were significantly lower compared to the diploid males (Table 5.1). In the case of the two $2n \times [2n+3n]$ crosses, theoretical (calculated on the basis of sperm cell

density) proportions of normal haploid (from diploid males) to presumably aneuploid spermatozoa (from the triploid males) were 1115:1 and 750:1 respectively. Under natural fertilisation conditions it seems unlikely that sperm from triploid males with low cell density and low or absent sperm motility could contribute to the fertilisation of eggs, but using the dry *in vitro* fertilisation technique the chances of fertilisation success may be determined by the presence of viable spermatozoa near the micropyle region. Since 1/9 (male number 1) and 1/5 (male number 2) of the offspring obtained in these crosses exhibited near-triploid genomes, they may have originated from aneuploid/near-diploid sperm collected from those triploid males, although the possibility of fertilisation by near-haploid sperm and spontaneous gynogenesis cannot be totally excluded.

Ueda *et al.* (1991) describe the generation of progeny by allotriploid trout males crossed with diploid rainbow trout females. Their chromosomal analysis revealed the presence of hyperdiploid and hypertriploid individuals among the eyed embryos, while the 5 fingerlings karyotyped exhibited a hypertriploid genome. They concluded that hyperdiploid embryos had originated from the fertilisation of normal (haploid) ova by 1.5n spermatozoa, and hypertriploid fingerlings were the result of unreduced (diploid) ova fertilised by 1.5n spermatozoa. Although spontaneous second polar body retention cannot be ruled out in our study, uniform diploid values observed in all 2n×2n crosses rather suggests that near-diploid spermatozoa from triploid males participated in amphimixis. The mechanism by which near-diploid spermatozoa could be produced in triploid testes is unknown, but spermatozoa in triploid testes are larger in size compared to normal haploid spermatozoa (Lincoln, 1981). A possible mechanism to produce diploid sperm in

triploid trout could proceed through polyploidization of the germ cell line to hexaploidy and then by reduction to diploid values via multipolar mitosis. The formation of multipolar spindles during early mitosis or during the first meiotic division, and the preferential elimination of chromosomes during spermatogenesis are plausible processes that may yield near-diploid gametes in a triploid gonad and have been described in other polyploids (Pera, 1975, Onishchenko *et al.*, 1979; Nakai *et al.*, 1991, Ohtani, 1993). In addition, some cyprinids and their hybrids are capable of modifying the meiotic division of their germ cells (Cherfas and Tzoy, 1984).

In agreement with previous data for diploid \times triploid carp crosses (Van Eenennaam *et al.*, 1990), the developmental rate of progeny obtained from $2n \times 3n$ matings was slower in comparison to diploid fish, as indicated by the late appearance of fry pigmentation and the delay in swim-up stage. Similar findings have also been reported in plaice (Lincoln, 1981) and another rainbow trout study (Lincoln and Scott, 1984). Similarly, the detection of polyploid hepatocytes in this study confirms previous work, as the liver is known to be one of the organs that includes a naturally polyploid cell population in fish (Brasch, 1980).

The presence of one precociously maturing male in the $2n \times 3n$ offspring illustrates the possibility that triploid trout may generate fertile sperm and thus perpetuate themselves. A potential, although highly restricted fertility of triploid males should be considered in the evaluation of the reproductive capacity of triploid salmonids, particularly if they are to be released into the wild.

CHAPTER VI

AN ANALYSIS OF CHROMOSOMAL PAIRING DURING MEIOSIS IN
DIPLOID AND AUTOTRIPLOID NILE TILAPIA,
OREOCHROMIS NILOTICUS

6.1 Summary

A cytogenetic analysis was carried out during the first meiotic prophase of diploid and triploid Nile tilapia, *Oreochromis niloticus* ($2n=44$). In order to obtain the desired genotypes, homogametic diploid and triploid populations were sex-reversed by oral administration of 17α -methyltestosterone at first feeding (XX^{MT} and XXX^{MT} males), and compared to heterogametic control and treated groups (XY , XY^{MT} , XXY and XXY^{MT} males). The pattern of chromosomal synapsis was investigated in all groups by transmission electron-microscope (TEM) analysis of synaptonemal complex (SC) spreads.

Administration of 17α -methyltestosterone resulted in an increase of the SC lengths in XY^{MT} males, but did not significantly alter the pattern of synapsis in comparison to XY controls. TEM analysis in diploid nuclei revealed the presence of 22 fully paired bivalents during pachytene in homogametic males. In heterogametic males (XY and XY^{MT}), an incompletely paired segment was frequently observed (24.2% and 28.6% of the cases examined, respectively) in the terminal region of the longest bivalent, suggesting the existence of a non-homologous segment in this chromosomal pair in the longest bivalent, and providing cytological evidence for the chromosomal basis of sex determination in *O. niloticus*.

TEM meiotic analysis in triploids revealed the presence of longer ($P<0.0001$) SCs in heterogametic (XXY and XXY^{MT}) than in homogametic (XXX^{MT}) genotypes. No significant differences were found in the extent of pairing between the three groups analysed; however, the nature of pairing was significantly different ($P<0.0001$) among groups, with a preferential bivalent + univalent association present in heterogametic XXY^{MT} males and absent in homogametic XXX^{MT} males. Based on these results, a model to explain the different progress in gametogenesis observed between male and female teleosts is discussed.

6.2 Introduction

Meiosis is the division during which homologous parental chromosomes are separated from each other to reduce diploid sets to the haploid level. Diploidy is restored through fertilisation by haploid cells; therefore meiosis is the necessary compensatory event to sexual reproduction. Pairing of homologous chromosomes at meiotic prophase is a precondition for their orderly segregation (Loidl, 1995).

The analysis of the fine configuration of chromosome pairing is possible by the study of the synaptonemal complex (SC), a protein scaffold which mediates in chromosomal pairing and recombination during the zygotene and pachytene stages of meiotic prophase I (Moses, 1956; Loidl, 1994; Egel, 1995; Hasenkampf, 1996). SC morphogenesis can be first visualised by the appearance of short stretches of protein cores called *axial elements* (AEs) along each pair of sister chromatids (Figure 1.1, A). Chromosome synapsis proceeds by the establishment of intimate associations between homologous AEs (usually near the telomeres), which once assembled into the SC are then referred to as *lateral elements* (LEs). The area between two LEs is called the *central region*, where densely staining, spheroidal structures called *recombination nodules* can be observed (Von Wettstein *et al.*, 1984; Figure 1.1, B). These nodules are thought to be the cytological visualisation of large protein complexes involved in the enzymology of recombination, since their frequency and distribution correspond to the frequency and distribution of crossovers (Carpenter, 1988). After pachytene, the SC proteins begin to disassemble and chiasmata are visible as sites of attachment between non-sister chromatids (Jones, 1987).

Although the precise role of the SC in recombination is the subject of active ongoing research, its analysis stands as a useful tool to investigate abnormal pairing

configurations associated with chromosomal differences between the parents, having been successfully used for the meiotic analysis of many plant and animal species, including teleosts (Foresti *et al.*, 1983; Wise and Nail, 1987; Lin and Yu, 1991; Oliveira *et al.*, 1995).

In triploid organisms, two possibilities have been identified for how sets of three homologous chromosomes can synapse (Von Wettstein *et al.*, 1984). An SC may connect only two chromosomes at any site, the third being excluded from synapsis (bivalent + univalent, II+I synapsis), or a triple SC can connect all three chromosomes along some distance or even along their whole length (triple pairing, III synapsis). In most organisms only II+I synapsis has been observed, whereas in a few only III synapsis occurs. In the triploid basidiomycete *Coprinus cinereus* (Rasmussen *et al.*, 1981), solanaceous plants (Sherman *et al.*, 1989), triploid domestic fowl (Comings and Okada, 1971; Solari *et al.*, 1991), triploid *Lolium multiflorum* (Thomas and Thomas, 1994) and triploid *Saccharomyces cerevisiae* (Loidl, 1995) both types of pairing have been reported in the same nucleus. However, III synapsis seems to be the exception; normally an SC is formed between only two LEs in a region and the three AEs compete for participation in the SC which results in one or more pairing partner switches (PPSs) between paired LEs. If crossovers occur to both sides of a PPS, the bonds between the chromosomes are stabilised and a pachytene trivalent is maintained as a metaphase I trivalent (Sybenga, 1975). Although previous data are not conclusive, the frequency of trivalent formation seems to depend on factors such as SC length, recombination frequency and number of chiasmata produced (Kuspira *et al.*, 1986; Loidl and Jones, 1986; Gillies, 1989; Chandley, 1993). Triploid organisms segregate the chromosomes of trivalents randomly in meiosis,

which leads to the production of aneuploid gametes. On fertilisation these produce zygotes with multiple trisomy, which are frequently inviable (Loidl, 1995).

The SC analysis in triploids has revealed a number of peculiarities. In many triploids (e.g. *Allium sphaerocephalon*, *Lolium multiflorum*, rye) there seems to be a drive in early pachytene to maximise pairing irrespectively of homology, presumably to increase the stability of unpaired AEs before the onset of chromosomal disjunction. Unpaired AEs engage in heterologous synapsis which results in the formation of complex multivalents, since often synapsis occurs between unpaired AEs of different trivalents (Loidl and Jones, 1986; Thomas and Thomas, 1994; Santos *et al.*, 1995). This process is termed *synaptic adjustment*. In addition, in some (but not all) triploids in which synaptic adjustment takes place, a second mechanism known as *pairing correction* operates to reduce the number of multivalents (including trivalents) by late pachytene. SCs in multivalents are partially dissolved and reformed by pairing correction so that bivalents and univalents are preferentially formed (Jenkins and Rees, 1991; Thomas and Thomas, 1994). A classical example is the elimination of multivalents and the formation of bivalents and univalents during prophase I in autotriploid female *Bombyx* (Rasmussen, 1977), which requires that SCs formed between homologous AEs are dismantled to eliminate multivalents. This is considered to be possible due to the achiasmatic nature of meiosis in female *Bombyx*. In other autotriploids the loss of multivalents may be because of insufficient chiasmata or the absence of crossovers between heterologous AEs (Thomas and Thomas, 1994). Such a mechanism for multivalent elimination is however absent in several triploids such as *Coprinus cinereus* (Rasmussen *et al.*, 1981), *Crepis capillaris* (Vincent and Jones, 1993) and *Lolium*

multiflorum (Thomas and Thomas, 1994), where most trivalents formed at meiotic prophase I are retained into metaphase I.

There are few reports on the meiotic behavior of chromosomes in autotriploid teleosts. In parthenogenetic triploid, all-female populations of *Poeciliopsis* (Cimino, 1972), premeiotic endoreduplication occurs which results in the formation of hexaploid oogonia. This even number of 6 homologous chromosomes allows for only bivalents to be formed during prophase I in primary oocytes. The resulting secondary oocyte regains triploidy through the second meiotic division. Chromosome synapsis during meiosis has also been studied in autotriploid males of rainbow trout (Oliveira *et al.*, 1995b). In this species the formation of SCs at zygotene occurs in II+I fashion, and the unpaired AEs engage in different synaptic configurations resulting in the formation of complex multivalents which remain in a closely restricted region of the nucleus. These multivalents are eliminated by a mechanism of pairing correction so that by the end of pachytene almost exclusively bivalents are observed, with a probable extensive heterologous synapsis involving the extra set of chromosomes.

In this Chapter an analysis of meiosis in diploid and autotriploid Nile tilapia was carried out in order to investigate possible differences in the pairing behavior of chromosomes that may explain the differential effect of triploidy on the reproductive development of male and female teleosts. This species has been selected because of the advantages offered by a fully documented reproductive, genetic and karyological background (Majumdar, 1984; Rothbard *et al.*, 1987; Alvendia-Casasuay and Cariño, 1988; Nakamura and Nagahama, 1985, 1989), fast growth and early puberty, an XX-XY system of sex determination (Mair *et al.*, 1991) and the ease with

which its ploidy and phenotypic sex can be manipulated (Hussain *et al.*, 1991; Mair and Santiago, 1994; Vera-Cruz and Mair, 1994). In addition, this is one of the few teleost species in which the technique for the analysis of the SC has been previously applied (Foresti *et al.*, 1993).

Previous reports on the effects of sex in the pairing behavior of chromosomes have shown pronounced differences in average SC lengths between males and females of a given species, with a substantial correlation to sex-specific recombination frequencies (Von Wettstein *et al.*, 1984; Jones and Croft, 1989). During pachytene, the SC is consistently longer in women than in men, and the recombination frequency and number of chiasmata are also higher in women (Bojko, 1983; Wallace and Hulten, 1985). Recombination frequencies in some teleost families (i.e. salmonids) are generally higher in females than in males (May and Johnson, 1989). On the basis of this background, it is hypothesised that sex-specific chromosome pairing behavior conditioned by structural and mechanical differences present during meiotic prophase I (length of the SC, number of chiasmata and frequency of trivalent formation) may explain the differences in degree of meiotic progression achieved by male and female triploids.

6.3 Materials and Methods

6.3.1 Experimental animals and design

O. niloticus ($2n= 44$) individuals used in this study derive from a Lake Manzala (Egypt) population which has been extensively characterised by mitotic and meiotic karyology, electrophoresis and recombination rates studies (McAndrew and Majumdar, 1983; Majumdar and McAndrew, 1986; Mair *et al.*, 1991). In preliminary studies of SC spreads, oocytes were less amenable than spermatocytes to the spreading process and yolk interfered with the staining method. Consequently, it was decided to use masculinised genetic females (neomales) as a source of XX and XXX genotypes (Mair *et al.*, 1991; 1997). An outline of the experimental design is presented in Figure 6.1. Two monosex populations were generated by fertilising pooled eggs collected from two mature *O. niloticus* females with pooled sperm collected from either two progeny-tested XX 'neomales' (to produce an all-female population) or two progeny-tested YY 'supermales' (to produce an all-male population: Myers *et al.*, 1995). Triploidy was induced in half of the eggs from both populations by an 8,000 psi, 2 min-long pressure shock applied 9 min after fertilisation (Hussain *et al.*, 1991). At swim-up stage, hatchlings were transferred from incubation jars to 4 L aerated aquaria and maintained in heated ($T= 27 \pm 2^{\circ}\text{C}$) static systems with daily water replacement under similar photoperiod (14L:10D) and density (15 fish/L) regimes. Both populations were randomly divided into three replicates each of control and treatment groups.

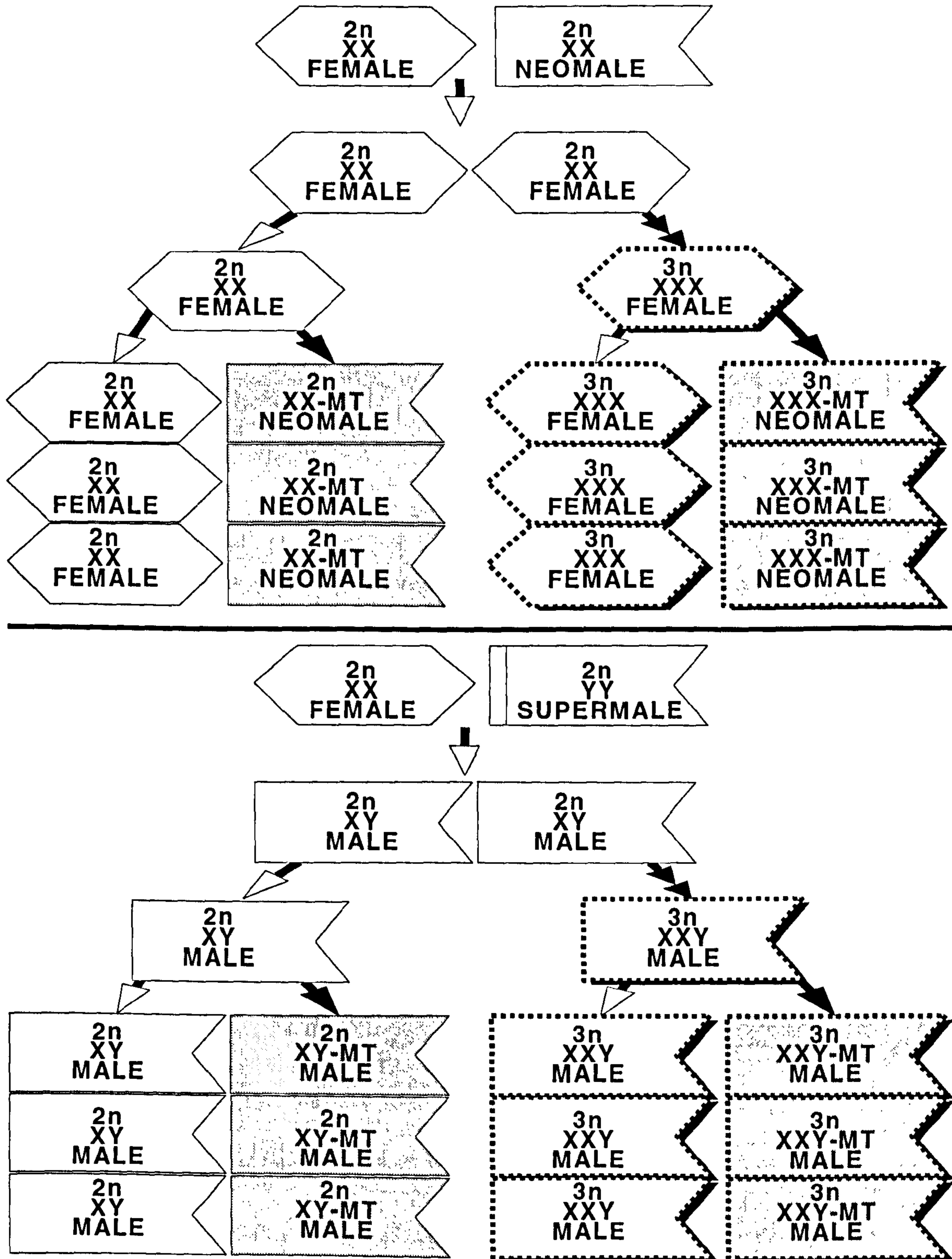


Figure 6.1. Origin, ploidy, presumptive genotypes and phenotypes of the all-female (top) and all-male (bottom) populations generated for the experiment. Double arrows signal triploidy induction, solid arrows indicate treatment with 17 α -methyltestosterone (MT). Control groups appear in white, treatment groups appear in grey, lines in boxes for triploid groups appear dashed.

Control groups were fed a finely ground (500-1000 μm) commercial broodstock diet three times per day; treatment groups were fed the same diet supplemented with 50 mg/kg of 17α -methyltestosterone (Sigma-Aldrich, Dorset, UK) during 30 days after first feeding (Mair *et al.*, 1991). All groups were transferred to 10 L recirculated tanks at 50 days post-fertilisation (p.f.) where they were held until sacrifice.

6.3.2 Ploidy and Sex determination

Ploidy was evaluated at 5 months p.f. in diploid and triploid control groups by measuring the nuclear size of red blood cells (RBC) with a Coulter Counter and Channeliser (Johnson *et al.*, 1984). Sex ratios were assessed by the aceto-carminesquashing technique (Guerrero and Shelton, 1974) at 5 months p.f. in diploid control groups.

6.3.3 Synaptonemal complex spreading

In order to monitor the degree of meiotic progression and to identify individuals suitable to be used as a source of abundant meiotic spreads, gonadal samples were collected weekly from one individual per replicate from each group. Treatment groups of the all-female population (presumed XX and XXX genotypes treated with 17α -methyltestosterone, XX^{MT} and XXX^{MT} thereafter) and control and treatment groups of the all-male population (XY , XY^{MT} , XXY and XXY^{MT} genotypes) were sampled starting at 10 weeks p.f.

The preparation of SC specimens was carried after the protocol of Foresti *et al.* (1993) with several minor modifications as follows. After overanaesthetising the males by immersion in a 0.01% benzocaine solution and recording their

morphometric parameters, the gonads were dissected, placed in a Petri dish containing 3 ml of Hank's saline solution (Sigma-Aldrich, Dorset, UK; adjusted to pH 8.5 with 1N NaOH) and carefully minced with two razor blades. The resulting cell suspension was aspirated with a Pasteur pipette and transferred into a sterile centrifuge tube placed in ice, where it was allowed to settle for 1 hr. The cell suspension, spreading medium (0.2% Lipsol[®] (LIP, Yorkshire, England) in distilled water adjusted to pH 8.5 with 0.01 M sodium tetraborate buffer) and a 0.2 M sucrose solution were added to treated histological slides in 1:2:2 proportions and gently mixed. Histological slides (Superfrost[®] BDH, Dorset, UK) had been thoroughly washed in soapy water, rinsed in distilled water, air dried and dipped in a 0.75% (w/v) solution of pioloform[®] plastic (Agar Scientific, Essex, UK) dissolved in reagent-grade chloroform. After 5 min exposure to the spreading medium, 1 ml of 4% paraformaldehyde fixative (buffered to pH 8.5 with sodium tetraborate) was added to each slide, and they were then left to dry overnight in a vertical position in a fume cupboard. The slides were rinsed and air dried before staining.

6.3.4 Staining and preparation of specimens for transmission electron microscopy (TEM)

Plastic-coated slides bearing surface-spread nuclei were coverslipped with 250 µm nylon cloth mesh (Plastok Associates, Merseyside, UK) and stained with a 50% silver nitrate solution at 50 °C for 50 min (Kodama *et al.*, 1980). Suitably spread nuclei were identified under the light microscope (Olympus Optical Co., Japan) and their position was recorded on the plastic coating using a permanent marker. The plastic was scored with a sharp razor blade along the edges of the

slide and floated off in distilled water. After 50 µm-mesh TEM copper grids (Agar Scientific, Essex, UK) were carefully positioned over the marks, a small piece of absorbent paper was dropped over the plastic film, and the resulting sandwich was quickly picked up and left to dry with the plastic film facing up. After drying, the TEM grids were detached from the plastic by scoring around their edge with a fine paint brush dipped in chloroform. The grids were examined at 80 kV using a Philips 301 TEM.

6.3.5 Synaptonemal complex analysis

Negatives from TEM pictures showing well-defined, unstretched and complete SC spreads were scanned at high resolution into a computer and analysed after enlarging the images to 10X-50X their original magnification using the Image Pro-Plus[®] image-analysis software (Media Cybernetics, Maryland, USA). Whenever possible a minimum of 4 meiotic nuclei at the pachytene stage were analysed per individual, and 6 individuals were analysed per group (two per each of three replications). The pictures were spatially calibrated using an internal standard, individual LEs and/or AEs were manually traced and total axial element length (TAEL) computed for every nucleus. Percent pairing values in a nucleus were calculated as:

$$\frac{\sum \text{Lateral Element Length}}{\text{Total Axial Element Length}} \times 100$$

The number of bivalents in triploid nuclei was counted manually. Only bivalents in which LEs were fully traceable from telomere to telomere were considered.

6.3.6 Statistical analysis

Parameter normality was assessed by the Kolmogorov-Smirnov normality test. Differences in TAEI length were investigated using a one-way ANOVA on logarithm-transformed values. Length measurements for the longest bivalent in diploids were expressed as a percentage of TAEI, arc-sine transformed and compared between groups by one-way ANOVA. Differences in percent pairing and number of bivalents between groups were investigated using a one-way ANOVA on arc-sine or square-root transformed values, respectively. Tukey's pairwise comparisons with a 95% joined level of confidence were constructed for mean ranking. In order to determine the degree of correlation between the parameters measured, the Pearson product moment or the Spearman ranking correlation analyses were used as appropriate (Neter *et al.*, 1990; Zar, 1996).

6.4 Results

6.4.1 Ploidy and sex ratios

Triploid yield in the all-female population was 94.59%. RBC diameter was $5.35 \pm 0.08 \mu\text{m}$ (mean \pm s.e.m) in the diploid control group (21 fish analysed) and $6.04 \pm 0.03 \mu\text{m}$ in the triploid control group (37 fish analysed). In the all-male population, triploid yield was 100%. RBC diameter was $5.56 \pm 0.09 \mu\text{m}$ in the diploid control group (10 fish analysed) and $6.25 \pm 0.11 \mu\text{m}$ in the triploid control group (27 fish analysed). Overall triploid yield was 96.87%.

Sex ratios were 100% female in the all-female population (30 fish sexed in diploid control) and 100% male in the all-male population (18 fish sexed in diploid control). No differences in sex ratios between replicates, undifferentiated or hermaphroditic gonads were observed.

6.4.2 Sample size and morphometric parameters

Table 6.1 summarises the number, age and morphometric parameters of the males included in the experiment by group. The required number of individuals with sufficiently abundant meiotic spreads was first obtained in diploid and triploid neomales from the all-female population (XX^{MT} and XXX^{MT} genotypes). They were considerable younger (5 months p.f.) and with a lower mean gonado-somatic index (<0.4%) than their counterparts in the all-male population. Individuals suitable for analysis in the all-male population were first detected in the diploid control group (XY, 7.3 months p.f.) followed by the XY^{MT} group (8.3 months p.f.) and the XXY control group (8.4 months post-fertilisation).

GROUP	MALE	AGE (months p.f.)	n° of nuclei	BW (g)	FL (cm)	GW (g)	GSI (%)
XX ^{MT}	1	5.1	5	26.8	11.5	0.09	0.32
	2	5.1	6	34	12.5	0.26	0.76
	3	5.1	5	29.2	11.5	0.06	0.19
	4	5.1	8	31.5	12.2	0.14	0.44
	5	5.1	7	28.9	11.5	0.08	0.27
	6	5.1	7	22.9	11	0.06	0.24
	mean ± s.e.m.		5.1	6.3	28.9±1.6	11.7±0.2	0.11±0.03
XXX ^{MT}	1	5.1	4	74.6	15.5	0.26	0.35
	2	5.1	4	63.6	15	0.30	0.47
	3	5.1	4	36.7	12.2	0.24	0.65
	4	5.5	6	67.3	15	0.11	0.16
	5	5.5	4	69.3	15	0.05	0.07
	6	5.5	4	55.7	14.5	0.09	0.16
	mean ± s.e.m.		5.3	4.3	61.2±5.5	14.5±0.5	0.17±0.04
XY	1	5.3	2	33.9	12.5	0.13	0.38
	2	6.0	5	35.3	12.5	0.22	0.62
	3	6.0	4	51.9	14	0.51	0.98
	4	8.4	2	83.1	17	1.06	1.28
	5	8.4	4	82.3	16.5	1.04	1.26
	6	8.4	14	67.7	16	0.92	1.36
	7	8.4	2	79.7	16	1.04	1.30
mean ± s.e.m.		7.3	4.7	62.0±8.2	14.9±0.7	0.70±0.15	1.03±0.15
XY ^{MT}	1	8.3	2	54.6	15	1.22	2.23
	2	8.3	3	45.6	14.7	1.67	3.66
	3	8.3	2	54.5	14.5	0.45	0.83
mean ± s.e.m.		8.3	2.3	51.6±3.0	14.7±0.1	1.11±0.36	2.24±0.82
XXY	1	8.4	1	72.6	16.5	0.78	1.07
	2	8.4	1	92.8	17.5	0.60	0.64
	3	8.4	1	79.8	16.5	0.63	0.79
mean ± s.e.m.		8.4	1	81.7±5.9	16.8±0.3	0.67±0.06	0.84±0.13
XXY ^{MT}	1	9.5	2	97.1	16.2	0.34	0.35
	2	9.5	3	108.4	16.7	0.44	0.41
	3	11.3	6	90.1	16.8	0.75	0.83
	4	9.5	5	119.6	18	0.9	0.75
	5	11.3	6	89.6	16	0.24	0.27
	6	11.3	5	102.6	17	0.9	0.88
mean ± s.e.m.		10.4	4.5	101.2±4	16.8±0.3	0.60±0.12	0.58±0.11
TOTAL	31		134				

Table 6.1. Genotype, number, age, number of nuclei analysed and morphometric parameters of the males and neomales included in the experiment. BW= body weight, FL= fork length, GW= gonadal weight, GSI= gonado-somatic index (GW/BW×100), s.e.m.= standard error of the mean.

Individuals suitable for meiotic analysis were only detected in the XXY^{MT} group at 10.4 months p.f. Only three males in the XY^{MT} group (7 nuclei in total) and the XXY control group (3 nuclei in total) yielded suitable SC spreads. A total of 134 nuclei collected from 31 males were included in the analysis.

6.4.3 *Synaptonemal complex analysis in diploids*

In the 78 spermatocytes where complete nuclei were examined, SC spreads contained 22 silver-stained bivalents at the pachytene stage of meiotic prophase I, according to the staging criteria set by Villagómez (1993) (Figure 6.2). Their lateral elements appeared as two well-differentiated parallel filaments spanning the bivalents from telomere to telomere, with a distinctly stained attachment plaque visible at both of their ends and surrounded by a lighter halo of chromatin. Although visible in a few cases (Figure 6.3), no clear structure was consistently revealed by the silver-staining technique in the central region of the SCs, and the kinetochores could not be consistently visualised in each bivalent. The spreads appeared frequently in close association with spermatozoa heads, but no obvious bouquet arrangement of the bivalents was observed (Figures 6.4 and 6.5). Equally, no broken lateral elements were observed, and chromosomal interlocking, although present in some cases, was rare. A SC karyotype and the mean lengths of the 22 fully-paired SCs for the XX^{MT} and XY genotypes arranged in decreasing order of size are presented in Figure 6.6. One of the bivalents is conspicuously longer than the other 21 in all nuclei analysed, measuring $15.07 \pm 0.47 \mu\text{m}$ in XX^{MT} , $14.42 \pm 0.66 \mu\text{m}$ in XY and $17.27 \pm 1.95 \mu\text{m}$ in XY^{MT} genotypes (mean \pm s.e.m.).

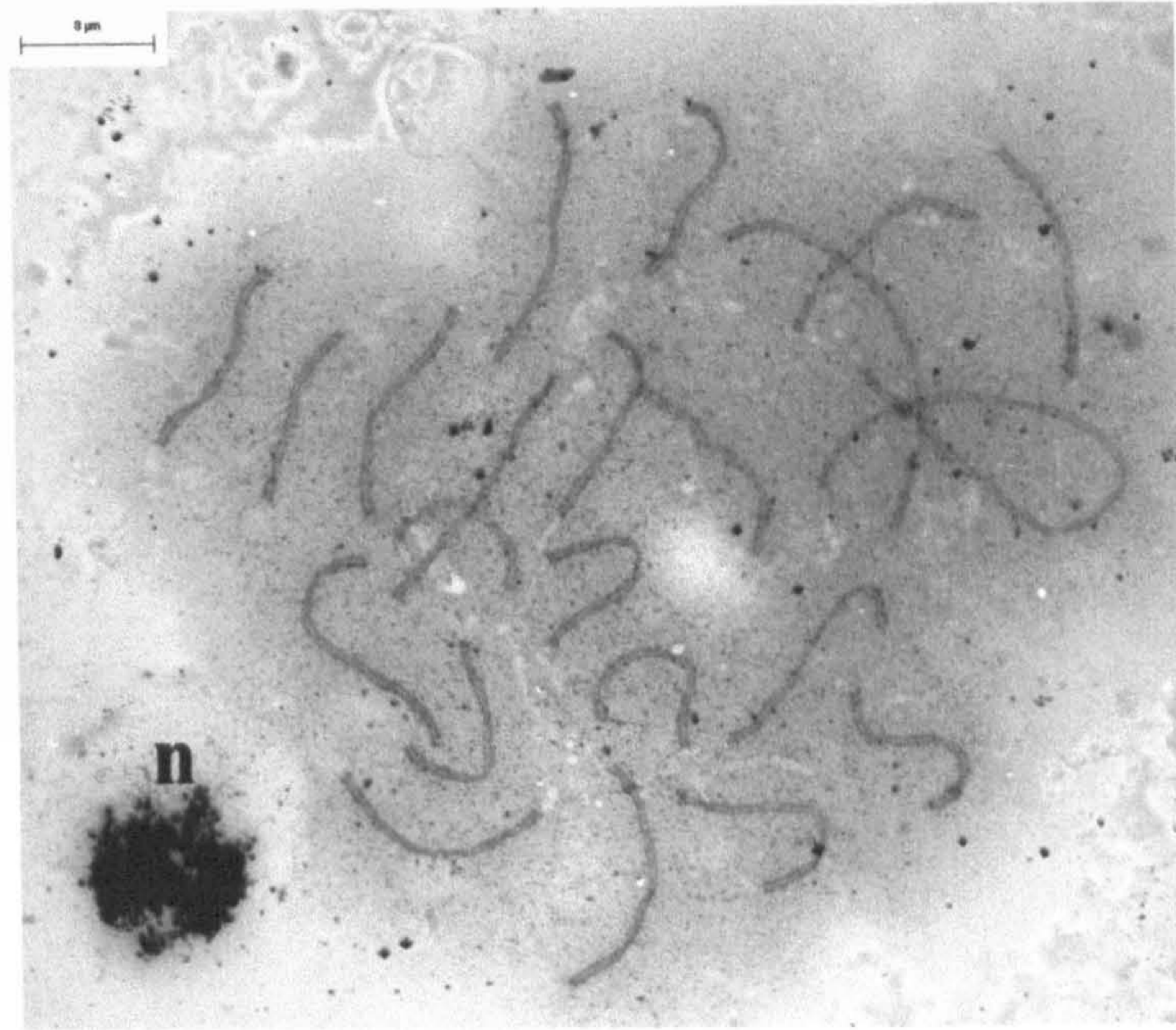


Figure 6.2. Complete synaptonemal complex spread in a male from the XY group. n, nucleolus. Scale bar= 3 μm.

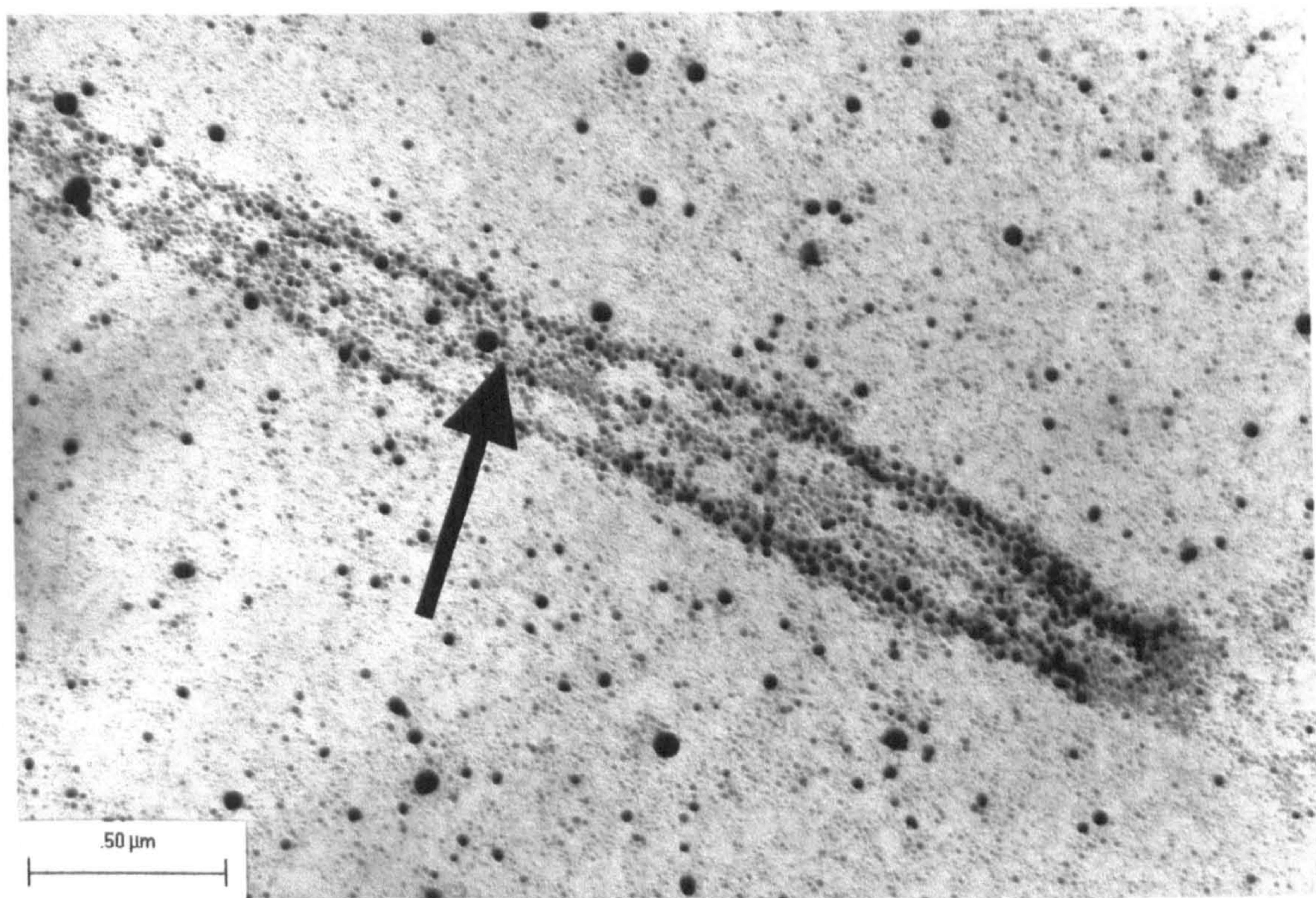


Figure 6.3. Detail of the lateral elements and central region (arrow) in the synaptonemal complex of a male from the XY group. Scale bar= 0.5 μm.

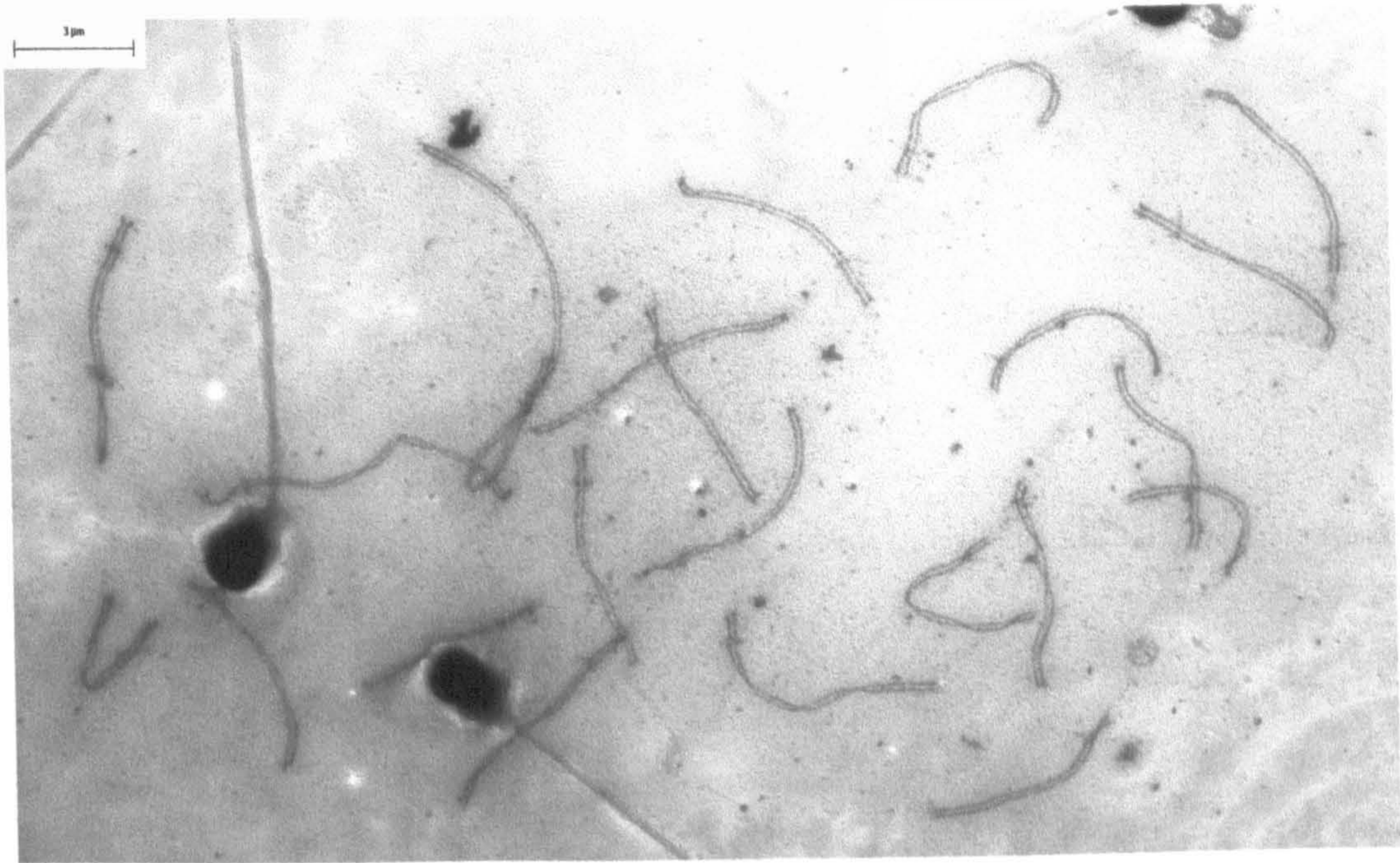


Figure 6.4. Complete synaptonemal complex spread in a male from the XY^{MT} group.
Scale bar= 3 μm.

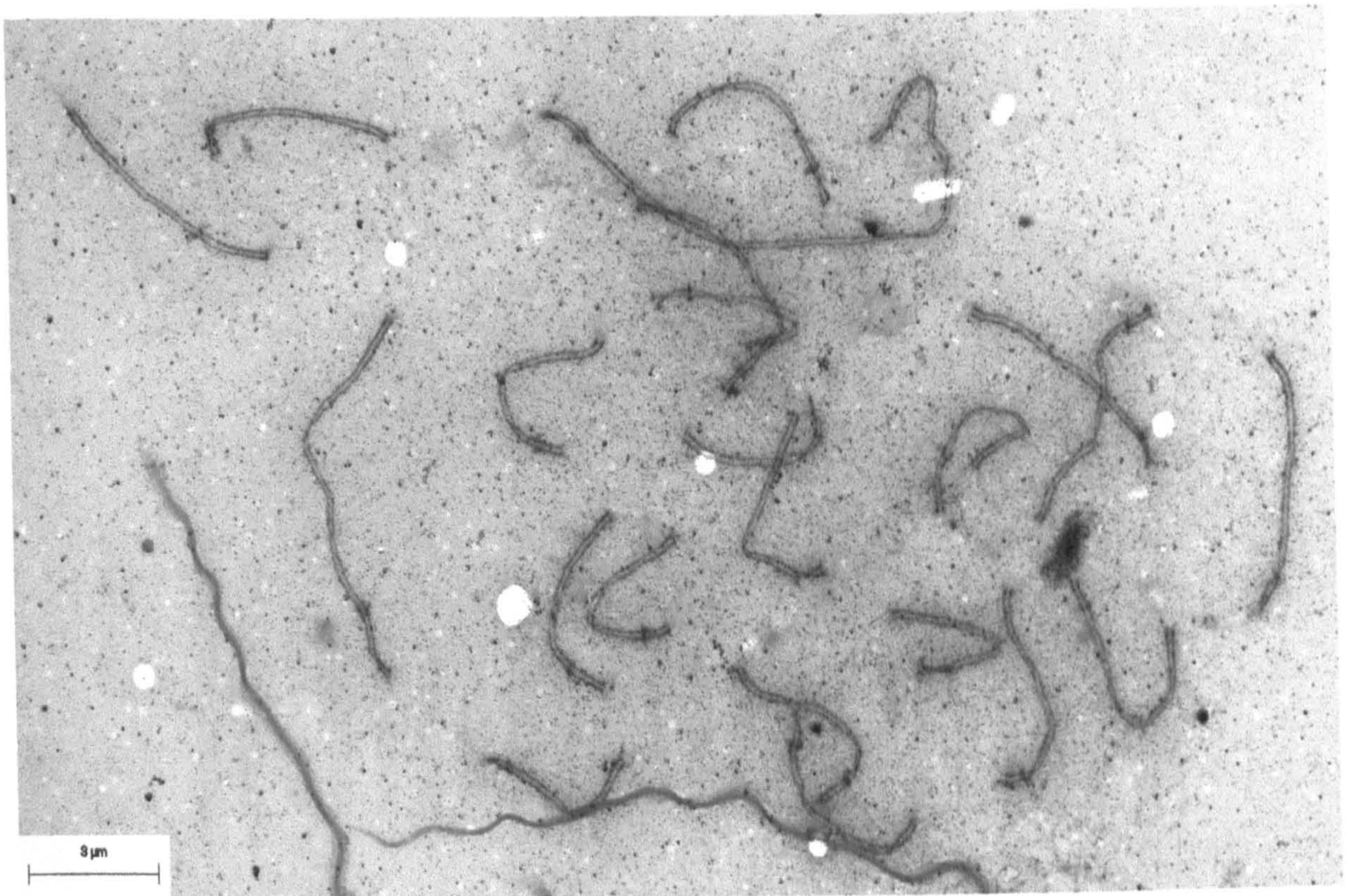


Figure 6.5. Complete synaptonemal complex spread in a male from the XX group.
Scale bar= 3 μm.

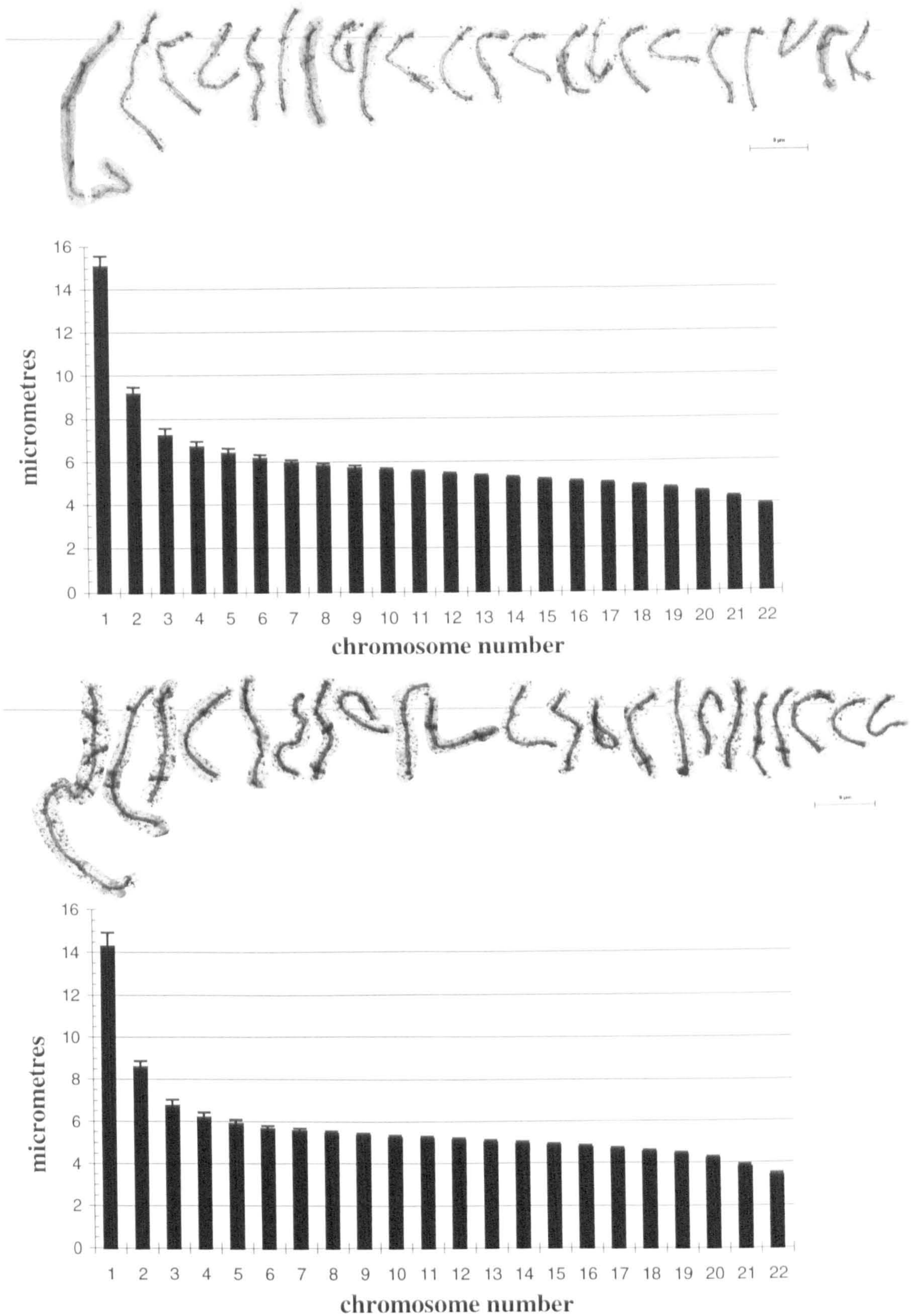


Figure 6.6. Synaptonemal complex karyotype and pooled mean lengths of the 22 fully-paired bivalents for the XX^{MT} neomales (n=37, top) and XY males (n=25, bottom) arranged in decreasing order of size. Lines in the karyotypes run across the presumptive location of the kinetochores. Magnification bars= 3 µm. Graph bars represent standard errors of the means.

These differences in length of the longest bivalent were neither significant between genotypes nor between males when expressed as a percentage of the total axial element length (TAEL).

The lateral elements of the 21 shorter bivalents paired in full along their length in all nuclei examined, presenting the normal SC morphology characteristic of other vertebrate groups. The AEs in the longest bivalent, however, showed some degree of unpairing in 8 nuclei of the XY group (24.2% of the nuclei analysed in XY genotypes, Figures 6.7 and 6.8), 2 nuclei of the XY^{MT} group (28.6% of the observations) and 1 nucleus of the XX^{MT} group (2.6% of the observations, Figure 6.9). Unpaired axial elements in the terminal region of the longest bivalent were observed in 4 of the 7 XY males and 2 of the 3 XY^{MT} males examined. Unpairing of the axial elements was restricted to the terminal and/or subterminal region of the longest bivalent in XY and XY^{MT} genotypes, while it affected the central and subterminal region of the longest bivalent in the single nucleus of the XX^{MT} genotype where unpairing was observed (Figure 6.9). Unpaired AEs in the XY genotype averaged 4.7 μm in length (32.6% of the average length of the longest bivalent in this genotype). When no region of unpairing was visible in the longest bivalent (97.4% of the cases examined in XX^{MT} males, 75.8% in XY males and 71.4% of the cases examined in XY^{MT} males) the lateral elements paired uniformly along its length (Figure 6.10). There was a significant, negative correlation between the length of the longest bivalent (expressed as a percentage of TAEL) converted to angles and the extent of pairing ($r = -0.432, P < 0.001$).

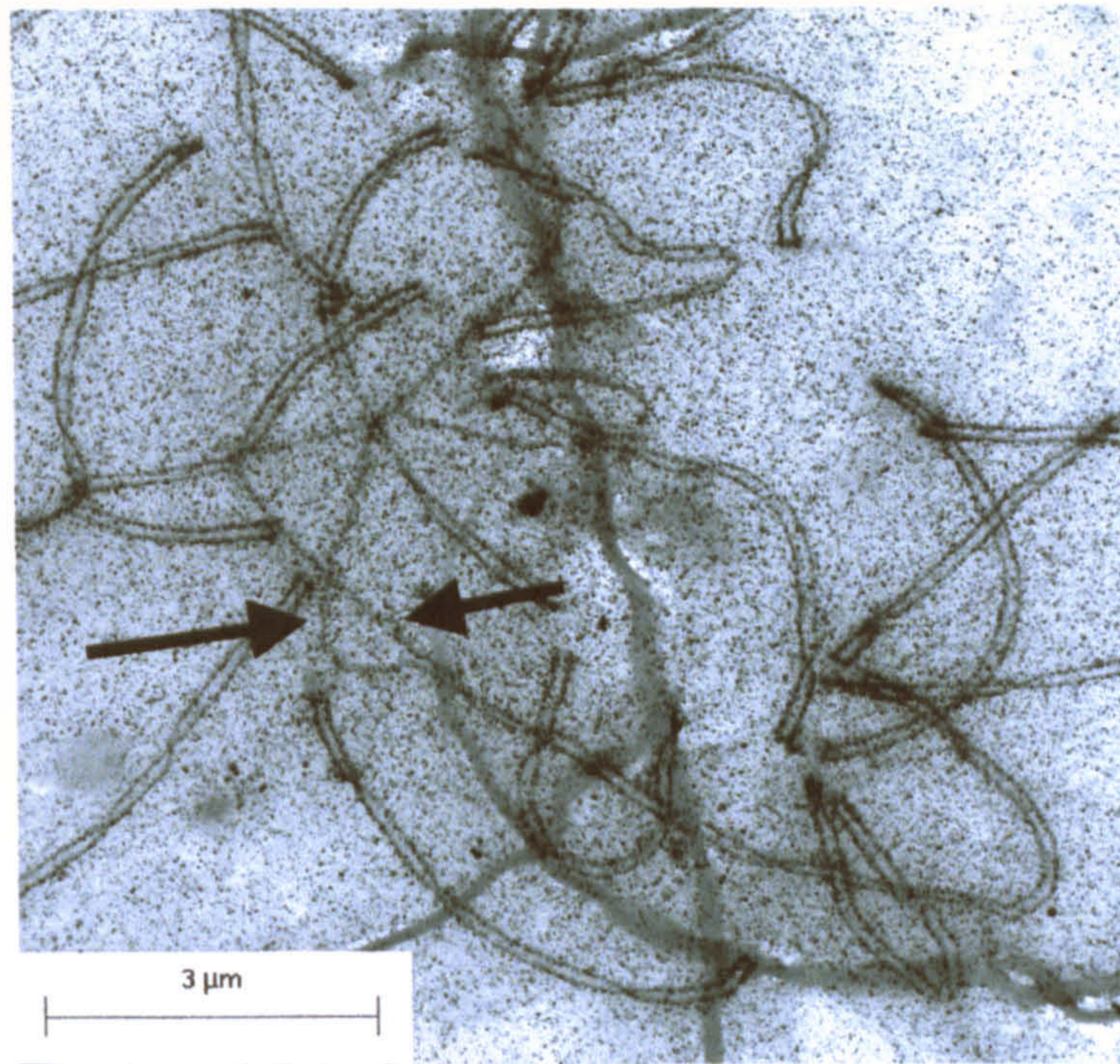


Figure 6.7. Unpaired axial elements (arrows) in the terminal region of the longest bivalent (XY male). Scale bar= 3 μm .

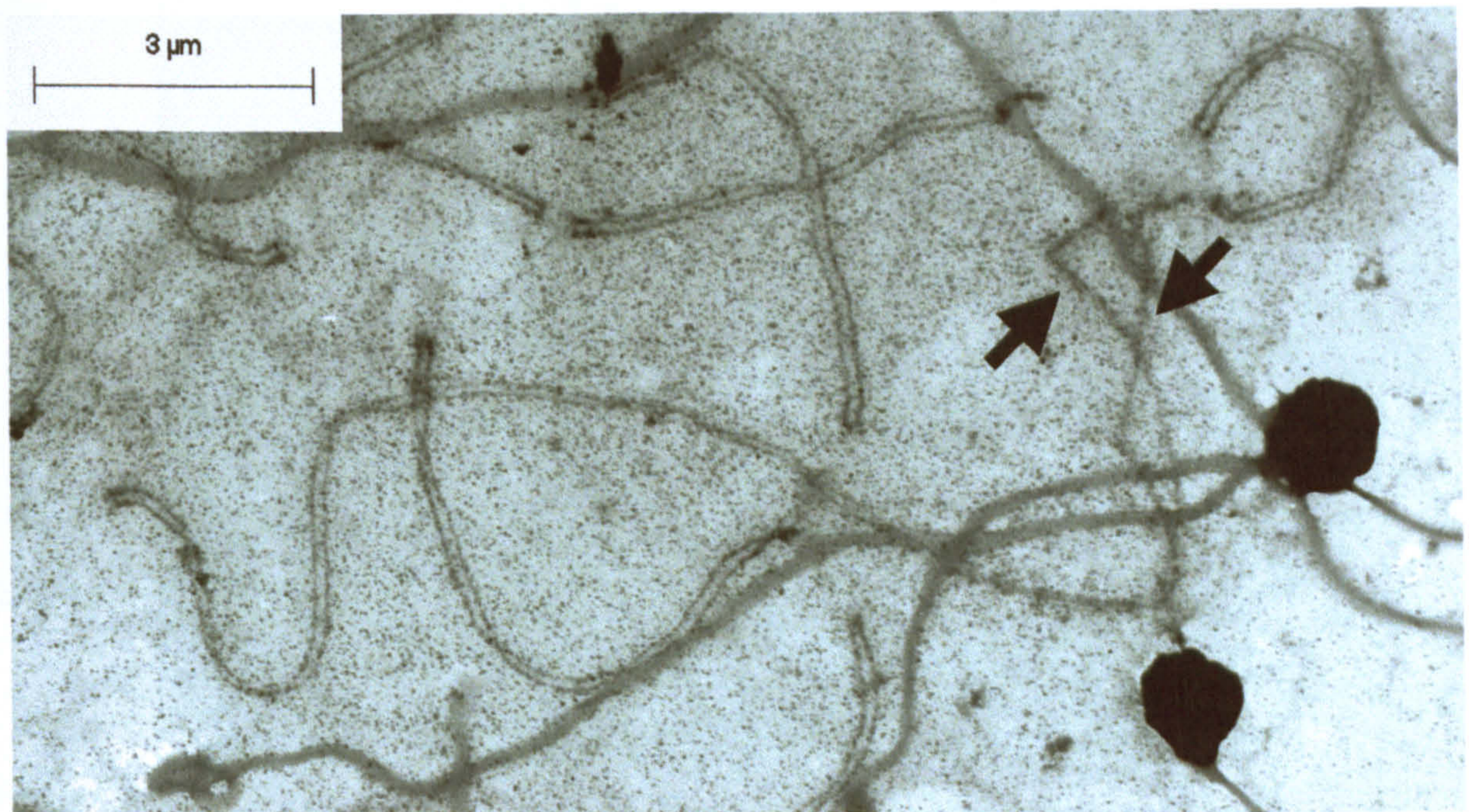


Figure 6.8. Unpaired axial elements (arrows) in the terminal region of the longest bivalent (XY male). Scale bar= 3 μm .

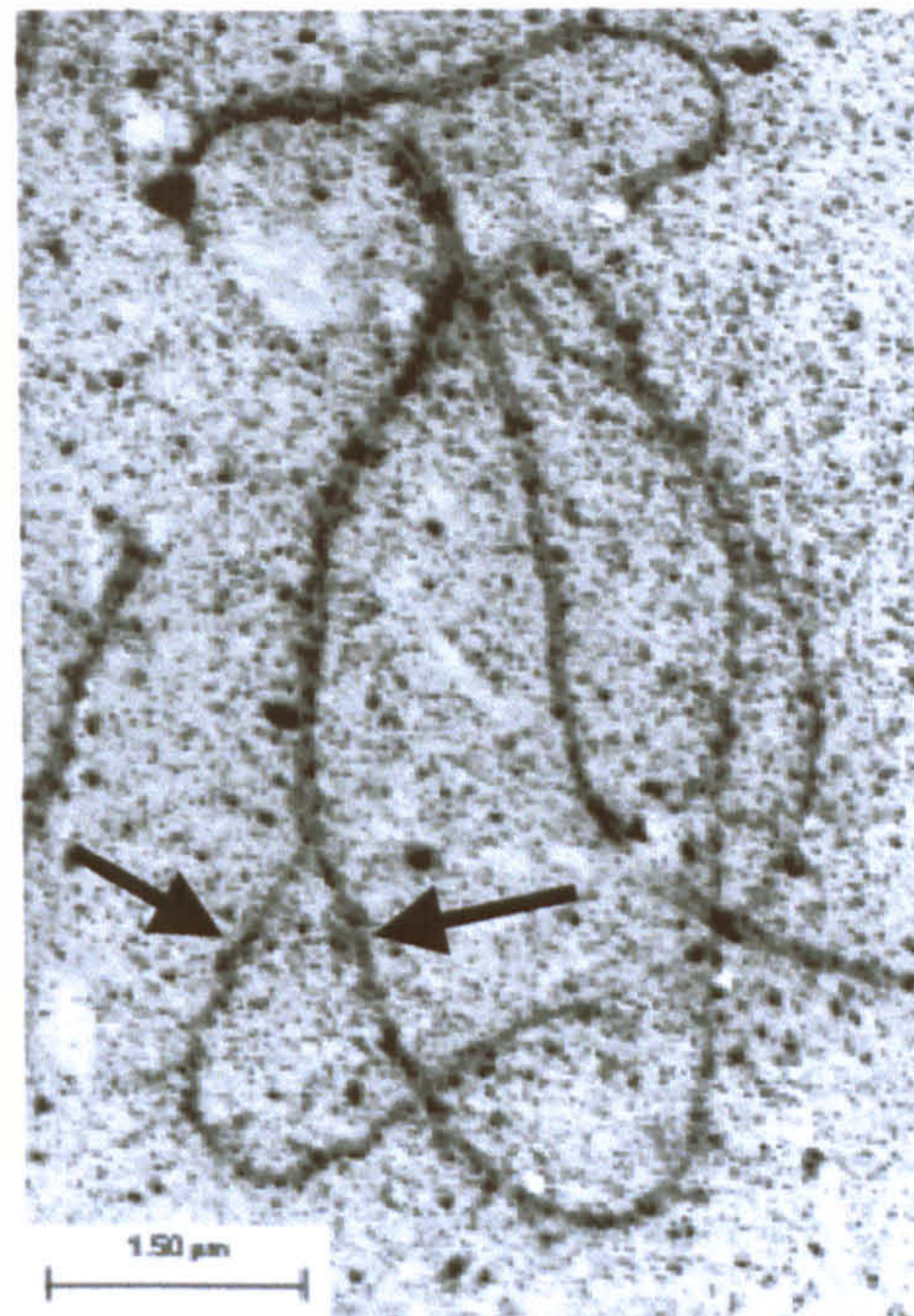


Figure 6.9. Unpaired axial elements in the central and subterminal region of the longest bivalent. The spread is from a male belonging to the XX^{MT} group.

Scale bar= 1.5 μm .



Figure 6.10. Fully-paired lateral elements in the longest bivalent of a male from the XY group. Scale bar= 2 μm .

Mean TAEI values were $264.04 \pm 5.34 \mu\text{m}$ for the XX^{MT} , $249.00 \pm 5.49 \mu\text{m}$ for the XY and 347.32 ± 20.61 for the XY^{MT} genotypes (mean \pm s.e.m.). Differences in TAEI mean values were significant between genotypes ($P < 0.0001$). Tukey's pairwise comparisons ranked TAEI mean values as $\text{XY}^{\text{MT}} > \text{XX}^{\text{MT}} = \text{XY}$. Differences in TAEI mean values were also significant between males ($P < 0.001$).

6.4.4 Synaptonemal complex analysis in triploids

Meiotic spreads in triploid genotypes appeared as dense, circularly-arranged SC aggregations in frequent association with bi-flagellate spermatozoa (Figure 6.11). Pairing generally comprised the telomeric regions in all genotypes, with the shortest SCs appearing as bivalents or trivalents and the longer SCs engaging in variable numbers of pairing partner switches (Figures 6.12 and 6.13). Almost the totality of pairing observed in the 56 SC spreads analysed in triploid groups occurred in II+I fashion, with a very low frequency of triple associations observed in all genotypes. Triple associations represented $0.10 \pm 0.07\%$ of the total axial element length in XXX^{MT} and $0.26 \pm 0.01\%$ of the TAEI in XXY^{MT} genotypes (mean \pm s.e.m.), and were not observed in the 3 nuclei from XXY genotypes analysed. When present, they appeared as short stretches of three parallel LEs invariably located in the telomeric regions (Figure 6.14).

A different pattern of synapsis was clearly evident in the different triploid genotypes examined. LEs engaged in multiple synapsis in XXX^{MT} and XXY genotypes, giving the spreads a complex, entangled appearance and making it difficult to identify individual trivalents/multivalents (Figures 6.15, 6.16 and 6.17).

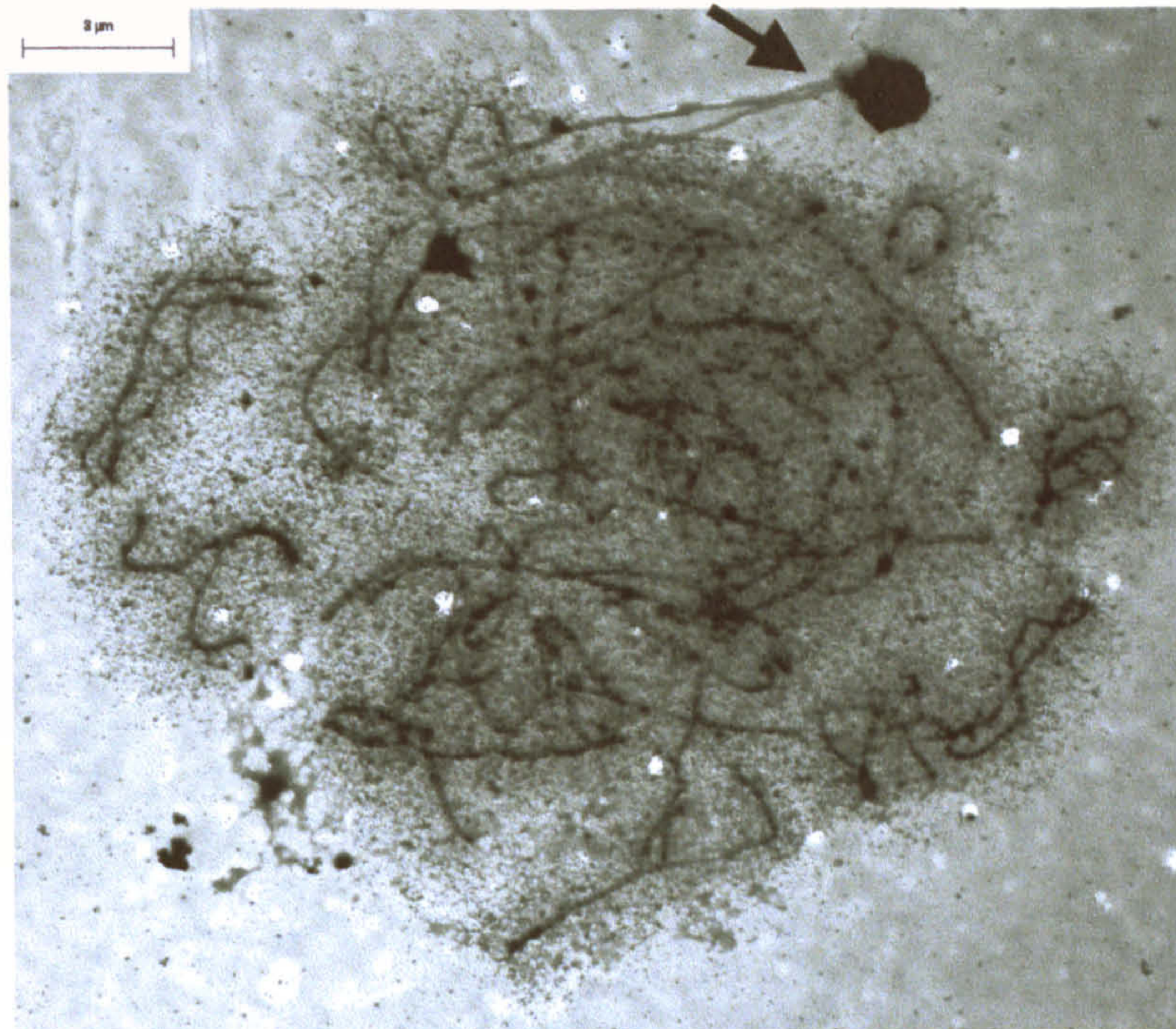


Figure 6.11. Full synaptonemal complex spread from a male belonging to the XXX^{MT} group. The arrow denotes the position of a biflagellate spermatozoon. Scale bar= 3 μm .

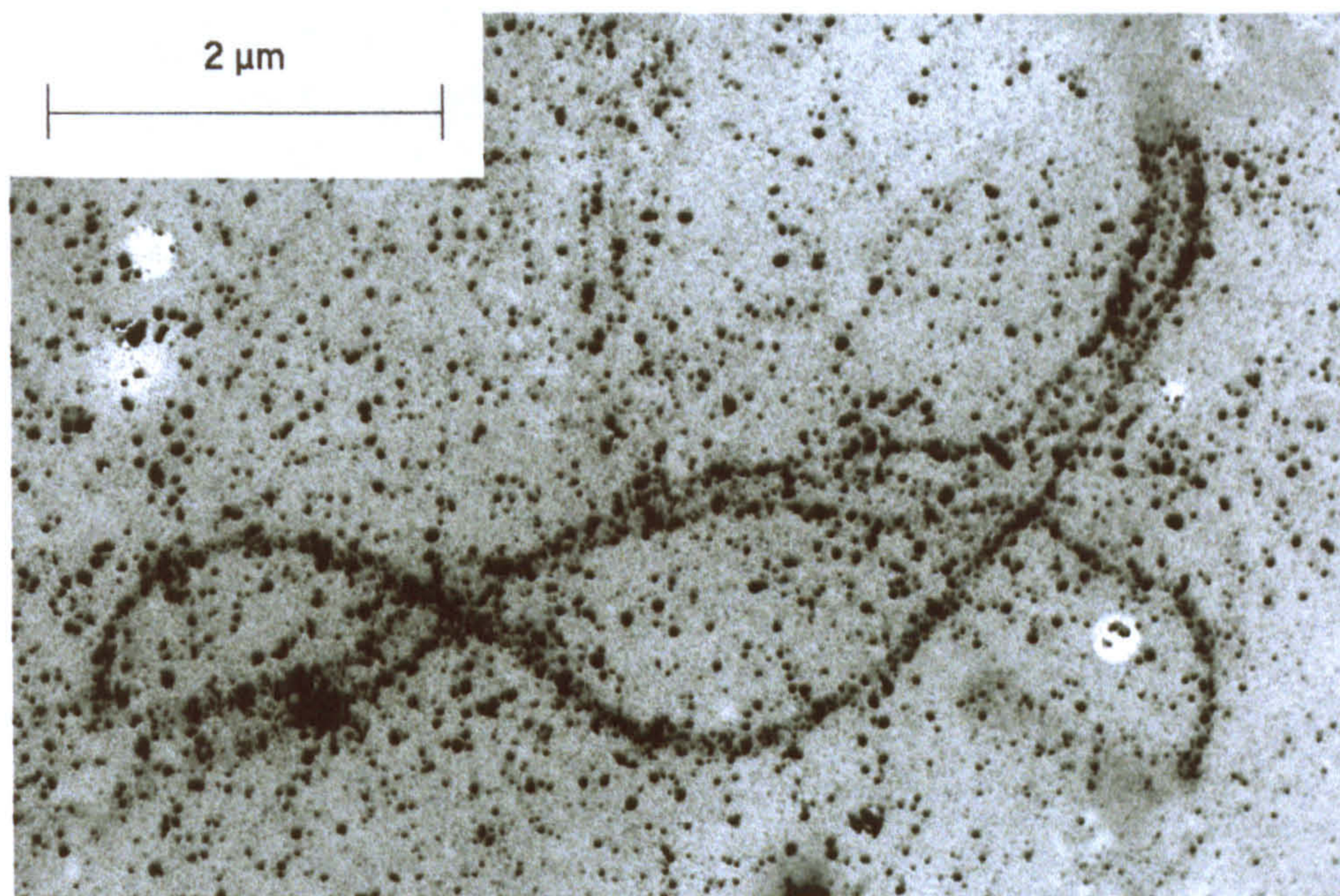


Figure 6.12. A trivalent from a nucleus of a XXY male. Scale bar= 2 μm .

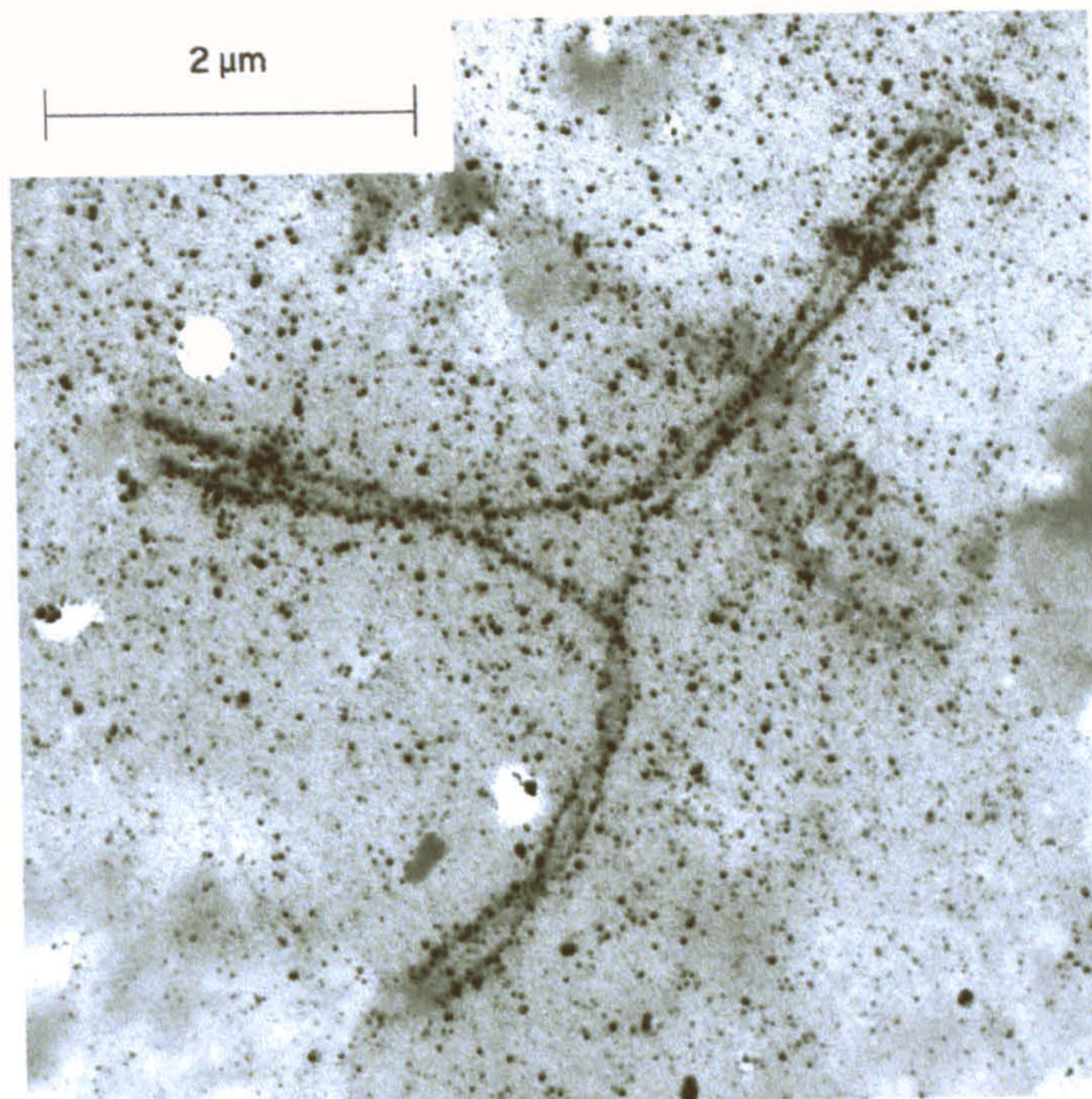


Figure 6.13. A trivalent from a synaptonemal complex spread in a male belonging to the XXY group. Scale bar= 2 μm .

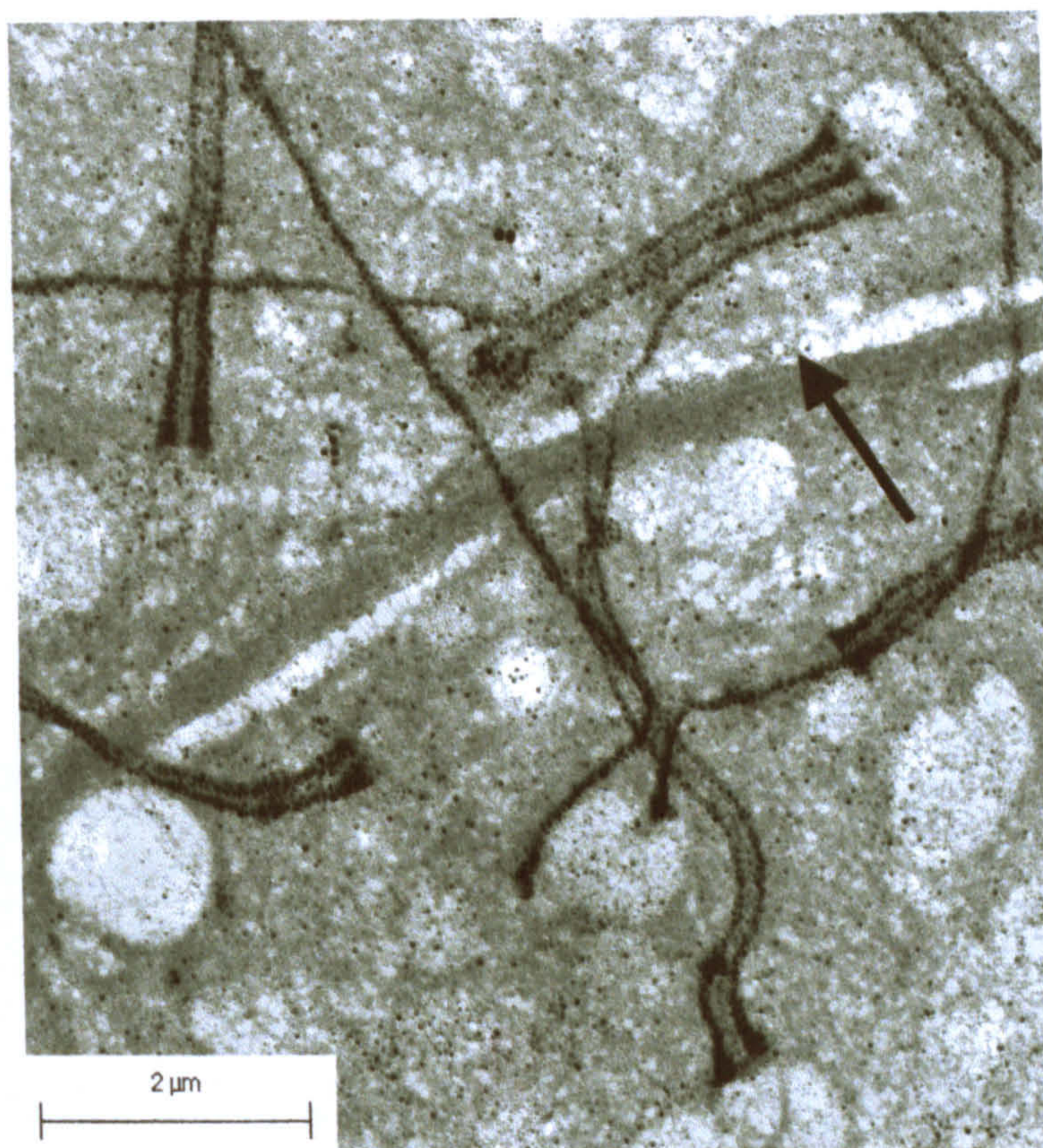


Figure 6.14. Triple association (arrow) in the terminal region of a multivalent. The spread is from a male in the XXX^{MT} group. Scale bar= 2 μm .

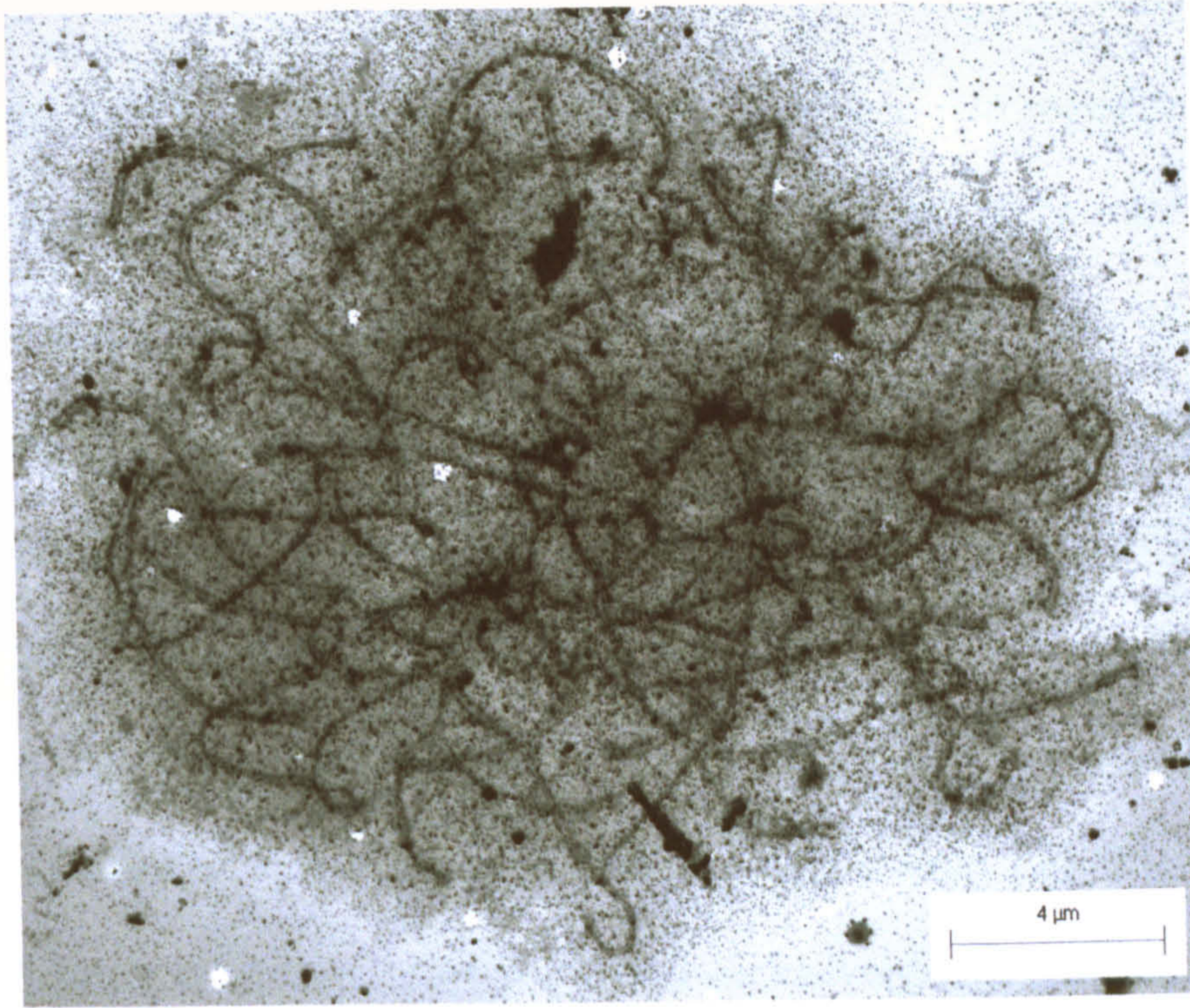


Figure 6.15. Full synaptonemal complex spread from a XXX^{MT} male. Scale bar= 4 μm.

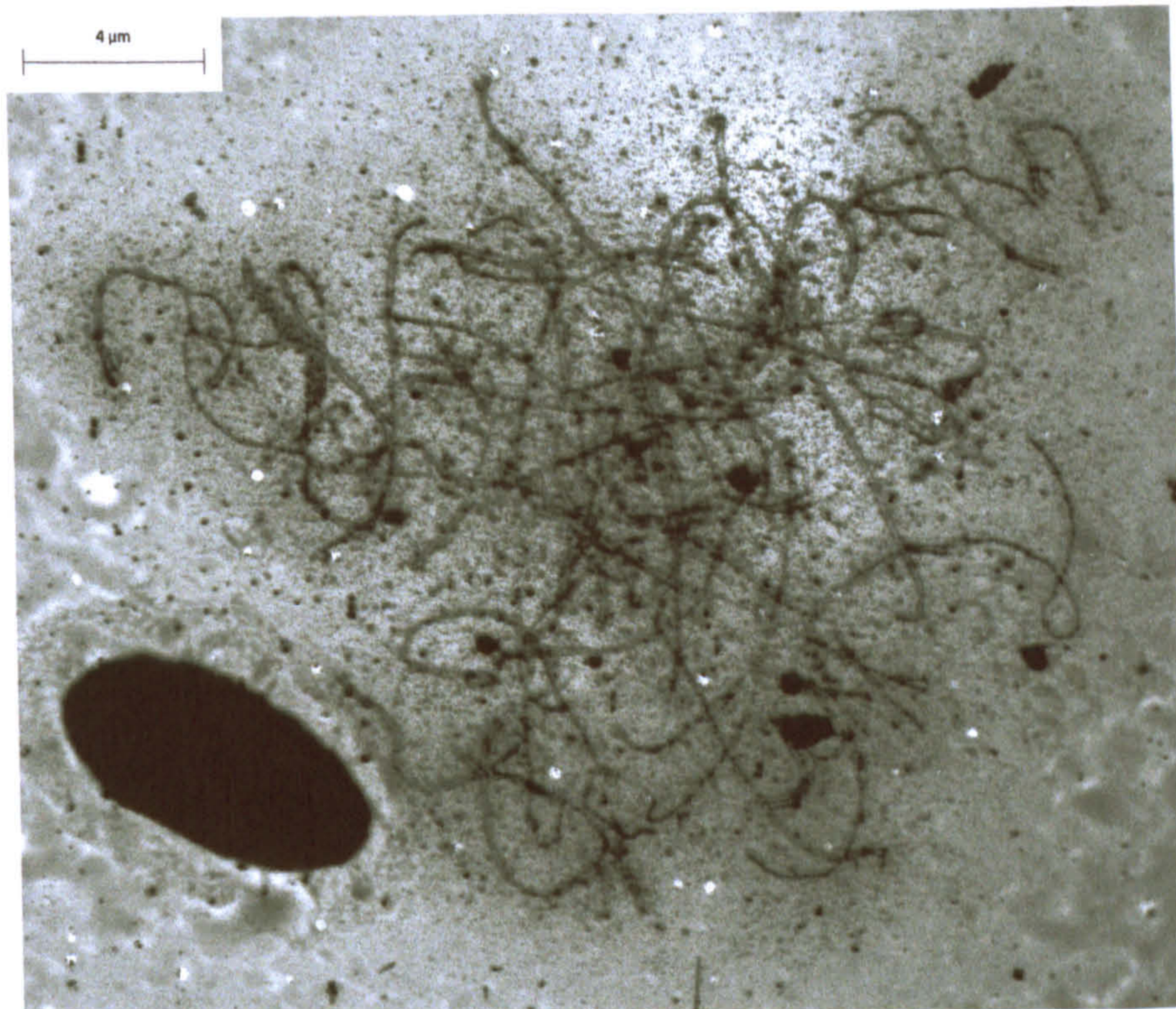


Figure 6.16. Full synaptonemal complex spread in a male belonging to the XXX^{MT} group. Scale bar= 4 μm.

In the XXY^{MT} genotype, however, the most common arrangement consisted of relatively high numbers (up to 16 in two nuclei of male 3) of fully-paired, well-defined bivalents located in the periphery of the spread, with one or several complex multivalents confined to a particular region of the nucleus, frequently in the centre of the spread (Figure 6.18, 6.19 and 6.20). Fully-paired bivalents in the XXY^{MT} genotype were frequently accompanied by the third unpaired AE running parallel at some distance to the SC formed by its two partners. Unpaired AEs appeared thickened and with noticeably darker staining intensity than paired LEs (Figure 6.21). One nucleus of the XXY^{MT} group (male 6) showed exclusively bivalent pairing, containing 66 fully-paired bivalents representing 132 chromosomes (the expected hexaploid ploidy level for *O. niloticus*) (Figure 6.22).

A summary of TAEL mean values, percent pairing (expressed as a percentage of TAEL) and the mean number of bivalents by genotype is presented in Table 6.2.

GROUP	n	TAEL (μm)	PAIRING (%)	N° of BIVALENTS
XXX^{MT}	26	405.6 \pm 16.8 ^a	58.72 \pm 0.02	3.7 \pm 0.4 ^a
XXY	3	543.3 \pm 38.5 ^b	56.04 \pm 0.04	4.7 \pm 0.5 ^a
XXY^{MT}	27	471.1 \pm 11.3 ^b	59.51 \pm 0.01	9.8 \pm 0.6 ^b

Table 6.2. Sample size, total axial element length (TAEL), percent pairing and mean number of bivalents observed in triploid genotypes. n= number of pachytene cells analysed. Values are mean \pm s.e.m.; different superscripts denote significantly different means.

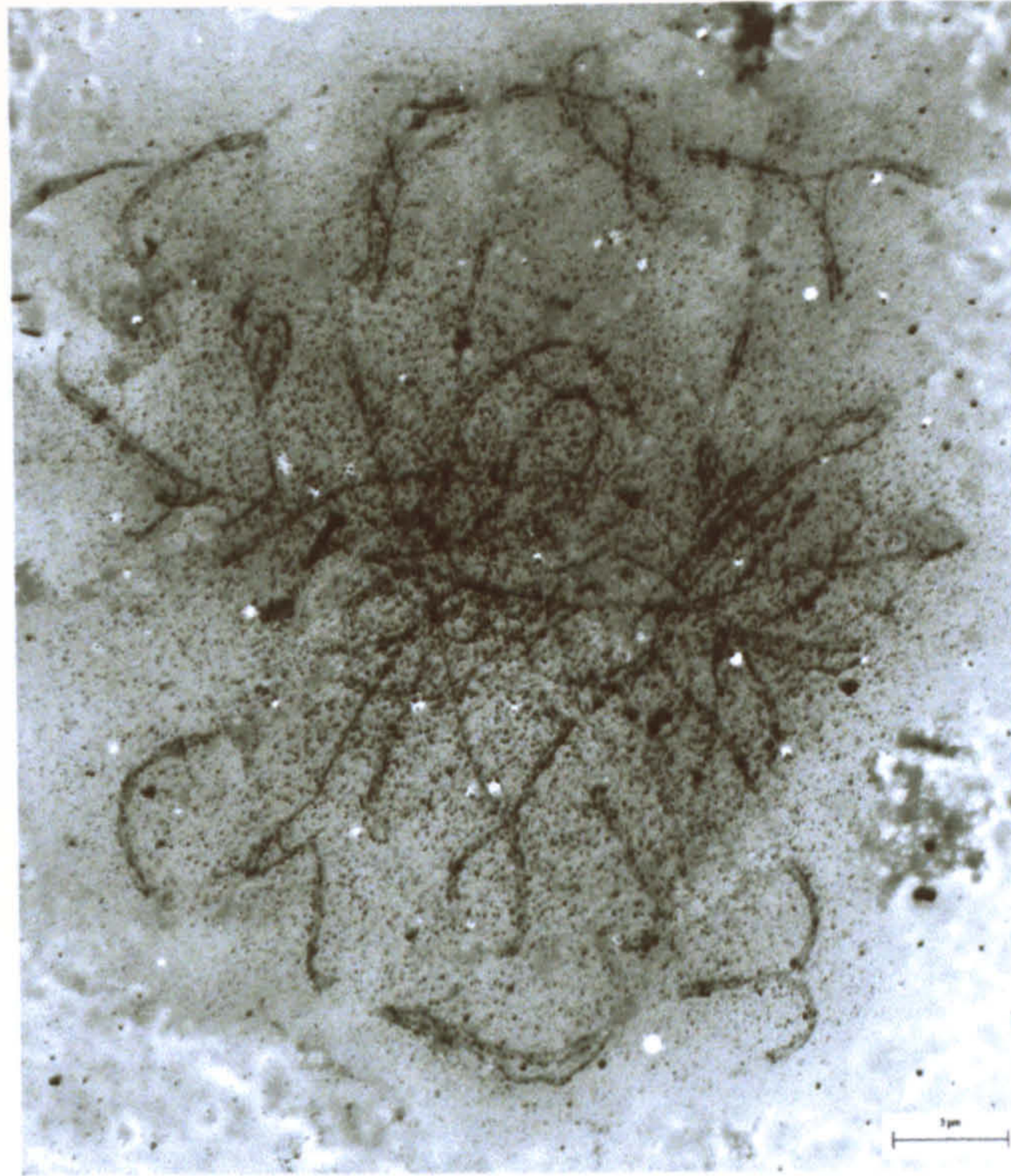


Figure 6.17. Full synaptonemal complex spread from a male in the XXY group. Scale bar= 3 μm.



Figure 6.18. Full synaptonemal complex spread from a male belonging to the XXY^{MT} group. Scale bar= 3 μm.

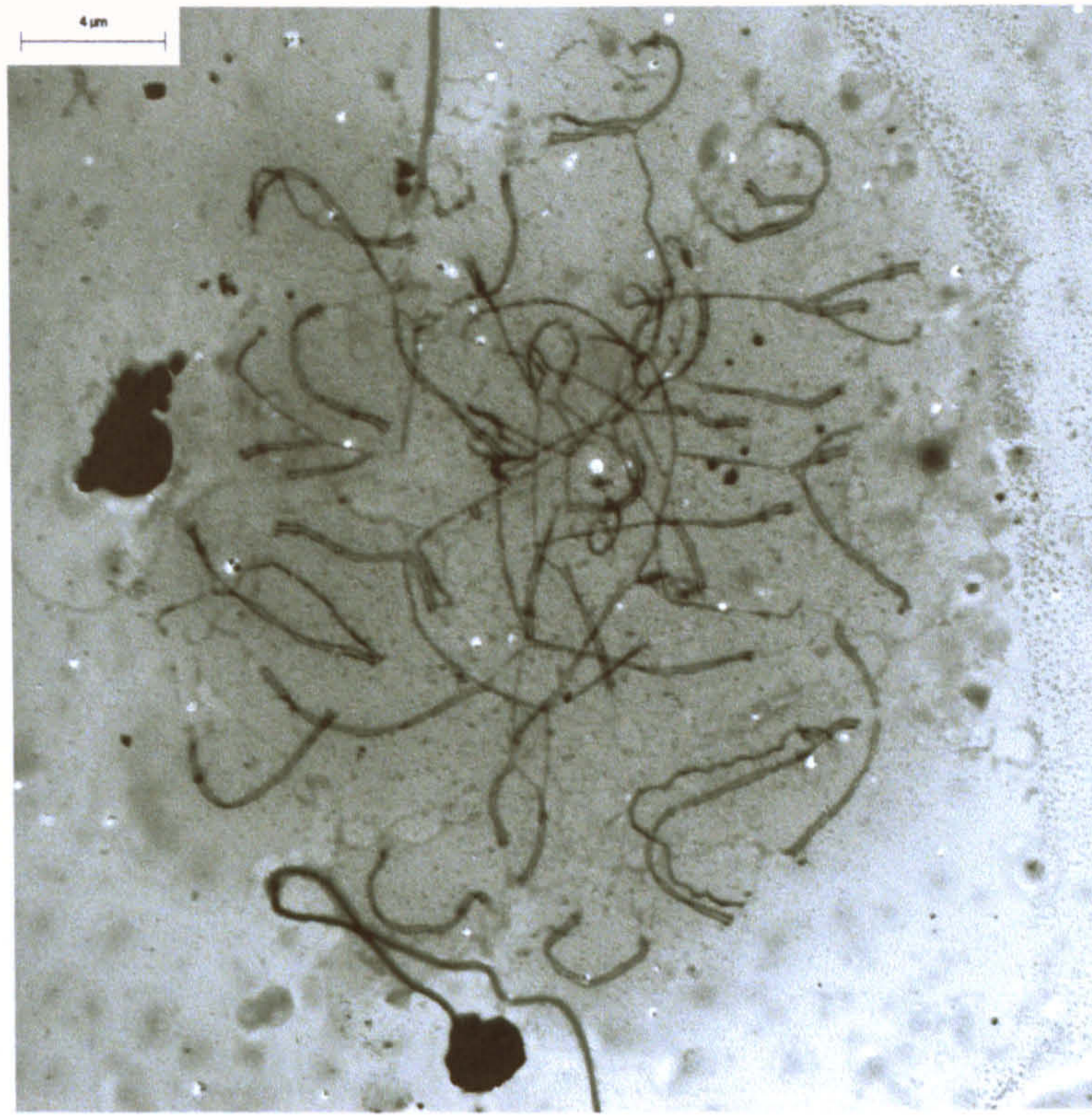


Figure 6.19. Complete nuclear spread in a XXY^{MT} male. Scale bar= 4 μm .

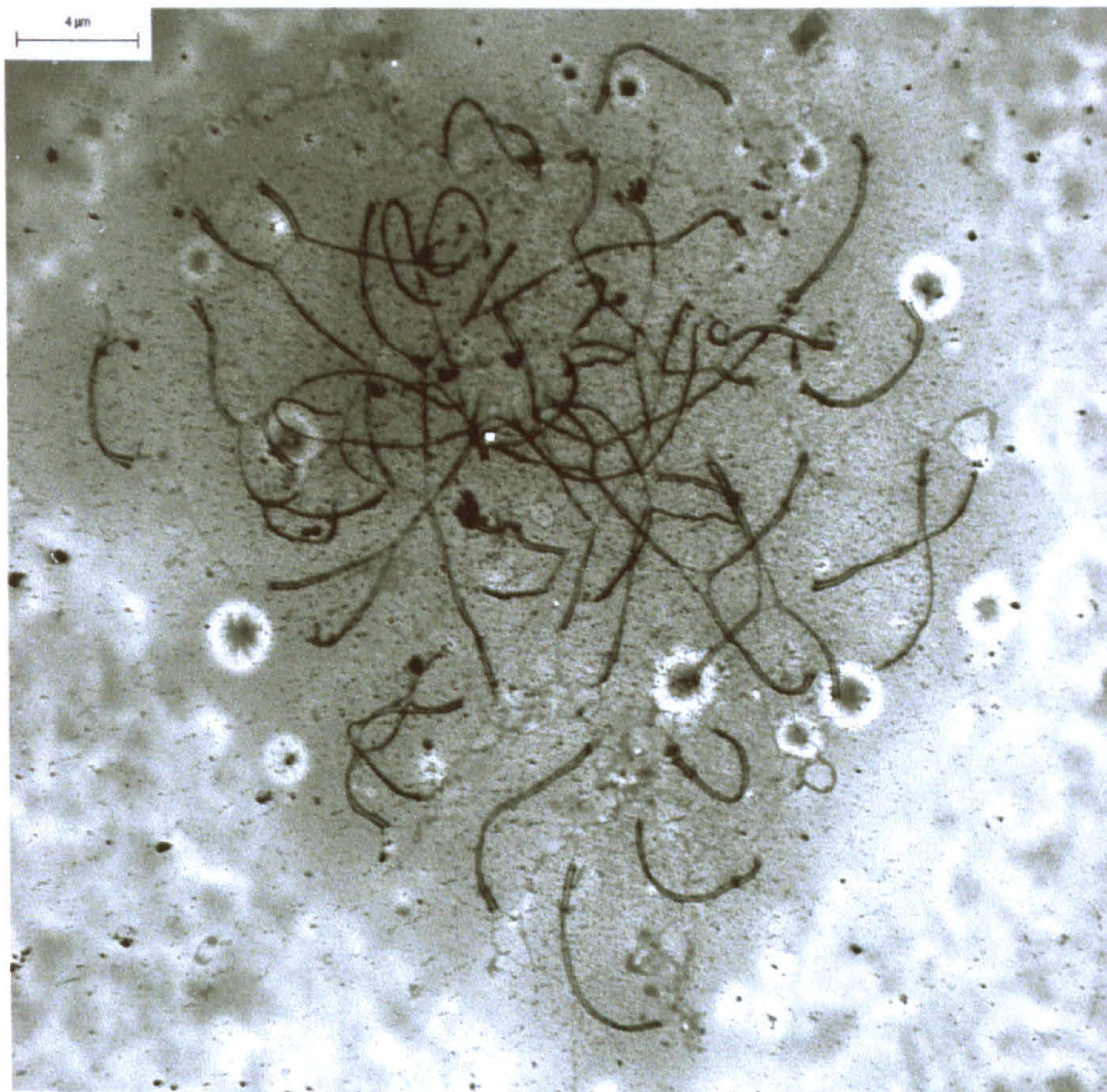


Figure 6.20. Complete synaptonemal complex spread from a male in the XXY^{MT} group. Scale bar= 4 μm .

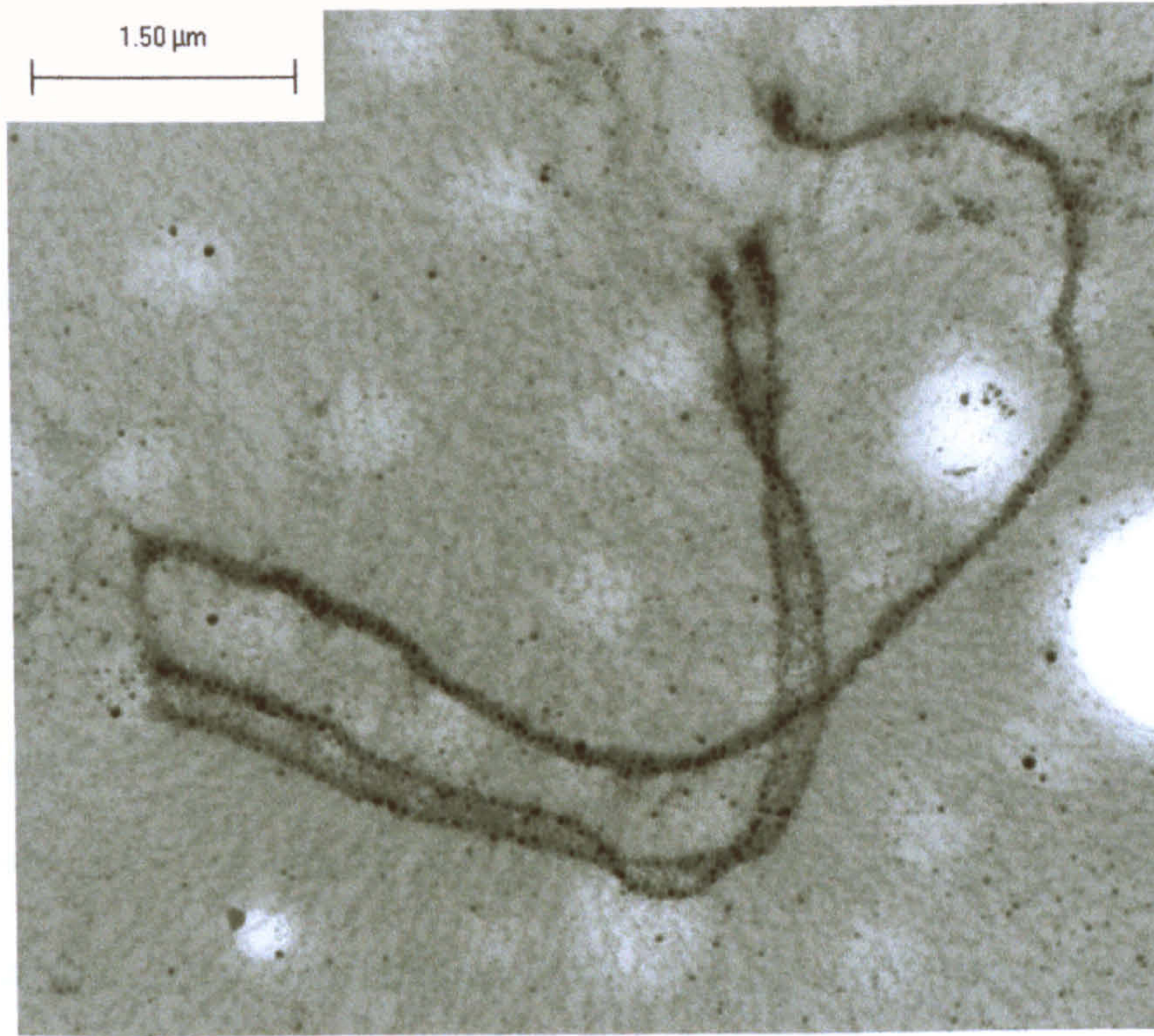


Figure 6.21. Example of bivalent + univalent pairing in a XXY^{MT} male. Notice the thickening and darkening of the unpaired axial element. Scale bar= 1.5 μ m.

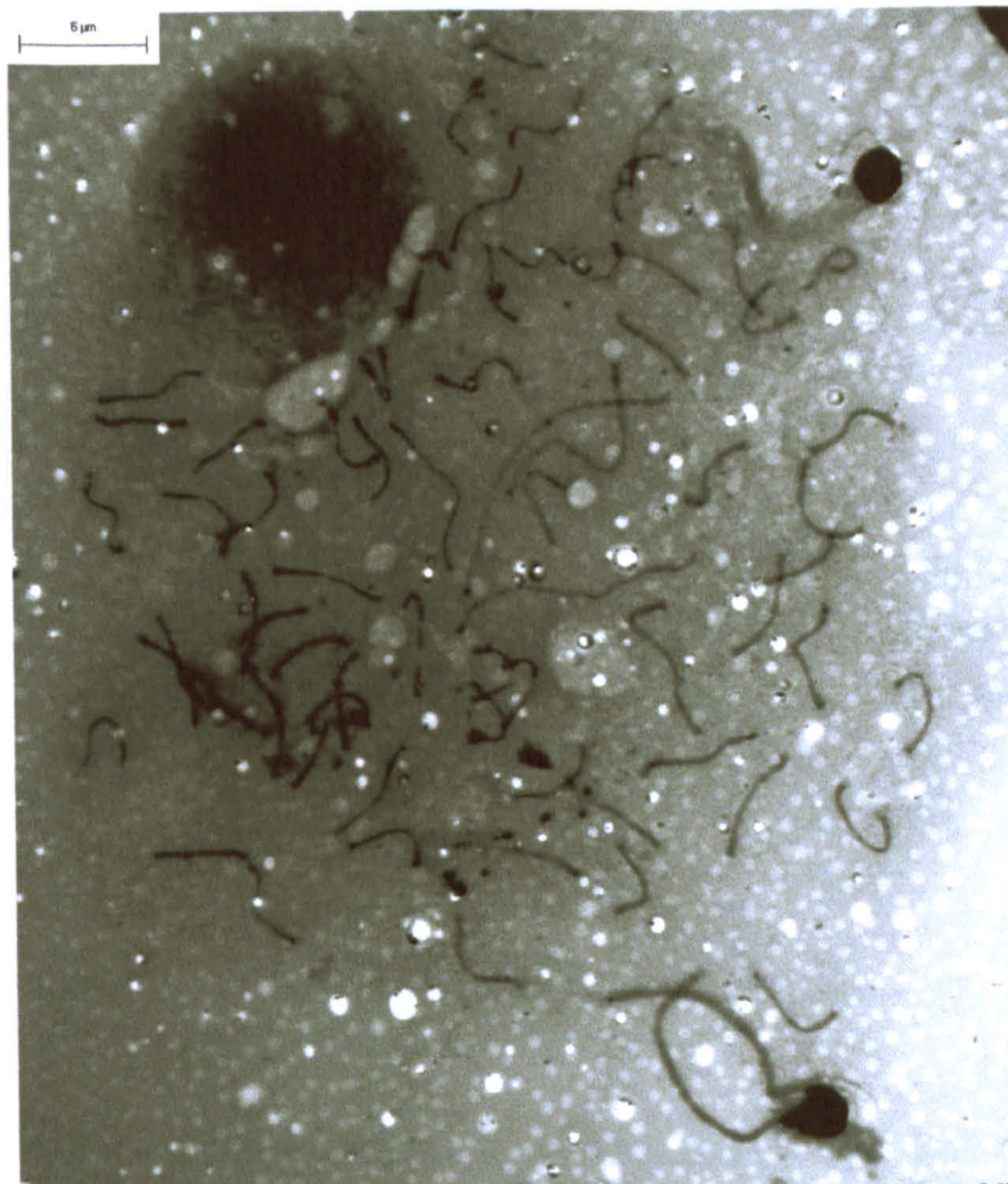


Figure 6.22. Synaptonemal complex spread from a male in the XXY^{MT} group. The number of bivalents in the spread is 66. Scale bar= 5 μ m.

Differences in mean TAEEL values were highly significant ($P < 0.0001$) between genotypes. Tukey's pairwise comparisons ranked mean TAEEL values as $XXY = XXY^{MT} > XXX^{MT}$. Differences in TAEEL values were significant ($P < 0.007$) between males, although they were limited to differences in TAEEL values between 3 of the 15 males included in the analysis. Differences in average percent pairing were not significant between genotypes or between males. The mean number of bivalents differed significantly between males ($P < 0.0001$) and genotypes ($P < 0.0001$). Mean ranking was $XXY^{MT} > XXY = XXX^{MT}$. There was no significant correlation between TAEEL values and percent pairing ($r = -0.08, P = 0.54$). There was a moderate, positive significant correlation between TAEEL values and the observed number of bivalents ($r = 0.308, P < 0.03$) (Figure 6.23).

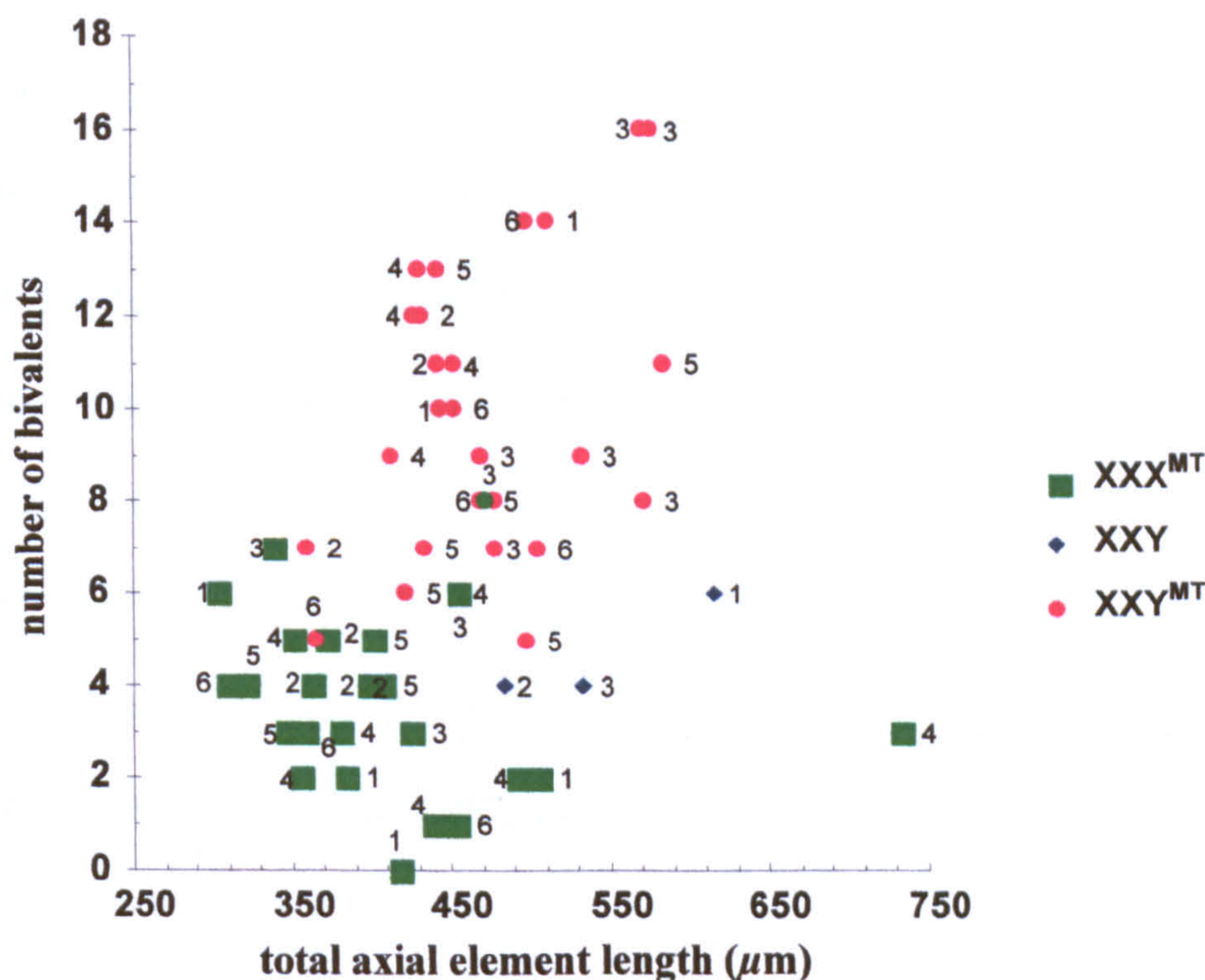


Figure 6.23. Total axial element length (TAEEL) and number of bivalents observed in the three triploid genotypes analysed. Data labels denote male number.

6.5 Discussion

6.5.1 Effects of 17α -methyltestosterone (MT) on chromosomal pairing

No previous reports have investigated the effects of oral administration of MT during gonadogenesis on chromosomal pairing in teleosts. In an analysis of the effects of testosterone, oestradiol and/or anti-androgenic substances on chromosomal pairing conducted in mice, the neonatal administration of oestradiol was found to have harmful effects on chromosomal pairing. Injection with oestradiol resulted in a high frequency (23.7% of pachytene cells examined) of SC anomalies such as asynapsis of the AEs, SC breakage, LE fragmentation and abnormal telomeric associations resulting in the formation of multivalents (Masumbuko *et al.*, 1992a, 1992b, 1993). Injection with testosterone enanthate during the neonatal period resulted in a reduction of testicular weight and a low frequency (2.6%, n=344) of similar SC lesions (Masumbuko *et al.*, 1993). These pairing anomalies, however, were not correlated with the reduction in testicular weight, and administration of testosterone to animals pre-treated with oestradiol significantly reduced the frequency of the SC lesions. The authors concluded that the observed effects of oestradiol were partially due to a testosterone deficiency induced by the oestrogen treatment. Other drugs with oestrogenic action, presumed to be operating by different primary mechanisms, also have the capacity to induce alterations in the synaptic process (Allen *et al.*, 1987; Goldstein, 1992; Mahmood and Vasudev, 1992; Sharpe *et al.*, 1993). The data from this work suggest that oral administration of MT after first feeding does not result in similar pairing alterations in *O. niloticus*. Mean testicular weight in XY^{MT} males was higher than in XY males, and very similar between XXY^{MT} and XXY males.

No significant alterations in SC morphology were observed in MT-treated males, and the pattern of pairing was not different between XY^{MT} and XY males, including a similar proportion (28.6% in XY^{MT} and 24.2% in XY males) of unpaired AEs in the longest bivalent. The relatively low number of pachytene cells examined in testosterone-treated males in this work (n=98), however, would limit the possibility of detecting such a low level of alterations.

Mean total axial element length values in *O. niloticus* MT-treated diploid males were significantly higher than in XY controls, but the failure to determine objective criteria for pachytene sub-staging in *O. niloticus* (i.e. kinetochore staining, presence of bouquet arrangements or nucleolar morphology), together with the small sample size in XY^{MT} (n=7) and XXY (n=3) genotypes makes the reliable assessment of the effects of MT administration on TAEL difficult. Furthermore, the biological significance of variations in the length of the SC is not obvious. Significant inter- and intra-individual variations in SC length are common among vertebrates (Table 6.3). It has been argued that the range of SC lengths observed in several plant species is a reflection of stage-related differences in chromosome length during pachytene (Anderson *et al.*, 1985). Progressive variation in SC length during pachytene has also been analysed in *Drosophyla* oocytes, where SC lengths were found to decrease during early to mid pachytene (Carpenter, 1979). In tomato (Stack and Anderson, 1986), *Zea mays* (Gillies, 1983) and *Crepis capillaris* the SCs undergo a progressive shortening throughout pachytene. If a similar pattern of variation is present in *O. niloticus*, the differences in SC length observed in this study between XY^{MT} and XY males may well reflect the attainment of a more advanced stage in pachytene in XY males than in XY^{MT} males, when most pairing had already been accomplished in both groups.

Species	Mean SC length (μm)	Range	Reference
<i>Homo sapiens</i>	231	1.30x	Holm and Rasmussen (1977)
<i>Homo sapiens</i>	258.7	1.36x	Solari (1980)
<i>Dendrocoelum lacteum</i>	94.6	1.45x	Jones and Croft (1989)
<i>Triticum aestivum</i>	1474	1.65x	Holm (1986)
<i>Cricetulus griseus</i>	116.2	1.67x	Moses <i>et al.</i> (1977)
<i>Felis catus</i>	207.6	1.71x	Gillies and Cowan (1985)
<i>Zea mays</i>	421	1.85x	Gillies (1981)
<i>Lilium longiflorum</i>	3149	1.93x	Stack <i>et al.</i> (1989)
<i>Lycopersicon esculentum</i>	207.4	1.94x	Sherman and Stack (1992)
<i>Secale cereale</i>	664.2	1.95x	De Jong <i>et al.</i> (1989)
<i>Schizophyllum commune</i>	23.2	2.38x	Carmi <i>et al.</i> (1978)
<i>Crepis capillaris</i>	163.4	2.53x	De Azkue and Jones (1993)
<i>Oreochromis niloticus</i>	132.5	2.33x	This study

Table 6.3. A summary of published data on synaptonemal complex (SC) length variation. Range= maximum/minimum value observed within a species.

The significant, negative correlation observed between the length of the longest bivalent and the extent of pairing in diploid genotypes seems to substantiate the view that increased extent of pairing through pachytene stages is accompanied by a shortening of the SC lengths. The higher SC lengths observed in XY^{MT} males would thus represent an earlier stage in the synaptic process in this group. The absence of significant differences in mean TAEL values between XXY and XXY^{MT} genotypes would also suggest that the variation in SC lengths observed in this study is within the normal range of pachytene stages in *O. niloticus*. Oral administration of MT may thus have had a delaying effect on the onset of the synaptic process in *O. niloticus*, but had not resulted in a significant alteration of the pattern of synapsis in the diploid specimens analysed in the present study.

6.5.2 Chromosomal pairing in diploids

The results from this work are in agreement with the mitotic karyotype reported by several authors for *O. niloticus* (Jalabert *et al.*, 1971; Arai and Koike, 1980; Nijhar *et al.*, 1983; Majumdar and McAndrew, 1986), with a diploid number of $2n=44$ corresponding to the 22 bivalents observed in this study. The number and morphology of the bivalents observed in this work are also in agreement with the previously reported SC karyotypes of *O. niloticus* (Lin and Yu, 1991; Foresti *et al.*, 1993), although the kinetochores or chromosomes bearing nucleolar-organising regions (NORs) could not be consistently identified, possibly as a result of technical limitations in the staining protocol.

A tentative classification of bivalents in this study identified four telocentric pairs (bivalents number 10, 19, 20 and 22), three subtelocentric-submetacentric pairs (bivalents number 2, 6 and 8) and 14 subtelocentric pairs (bivalents number 1, 3, 4, 5, 7, 9, 11, 12, 13, 14, 15, 16, 17, 18 and 21, Table 6.4). Lin and Yu (1993) identified one telocentric pair (bivalent number 1), four submetacentric pairs (numbers 6, 8, 15 and 19) and the remaining seventeen pairs of subtelocentrics. Majumdar and McAndrew's karyotype (1986) identified 1 metacentric pair (number 6), 9 pairs of submetacentrics (numbers 3, 5, 7, 8, 11, 12, 14 and 15), seven pairs of subtelocentrics (numbers 1, 2, 9, 17, 20, 21 and 22) and the remaining five pairs of submetacentric-subtelocentrics. It appears that although most or even the totality of (with the possible exceptions of bivalents number 6 and 8) the chromosomal pairs in *O. niloticus* are subtelocentric/submetacentric, the resolution of the analyses performed to date in this species cannot reliably discriminate between telocentric, subtelocentric or submetacentric chromosomes.

Chromosome number	Chromosome type			
	M.&M.	L.&Y.	This study	Consensus
1	st	t	st	st
2	st	st	st-sm	st
3	sm	st	st	st
4	sm	st	st	st
5	sm	st	st	st
6	m	sm	st-sm	sm?
7	sm	st	st	st
8	sm	sm	st-sm	sm
9	st	st	st	st
10	st-sm	st	t	st?
11	sm	st	st	st
12	sm	st	st	st
13	st-sm	st	st	st
14	sm	st	st	st
15	sm	sm	st	sm
16	st-sm	st	st	st
17	st	st	st	st
18	st-sm	st	st	st
19	st-sm	sm	t	sm?
20	st	st	t	st
21	st	st	st	st
22	st	st	t	st

Table 6.4. A comparison of previous meiotic karyotypes with the tentative chromosomal classification considered in this study for *O. niloticus*. M.&M., Majumdar and McAndrew (1986); L.&Y., Lin and Yu (1993). m, metacentric; sm, submetacentric; st, subtelocentric; st-sm, subtelocentric/submetacentric; t, telocentric.

6.5.3 The chromosomal basis of sex determination in *Oreochromis niloticus*

Perhaps the most significant observation of this study in diploid genotypes is the presence of unpaired segments in the terminal region of the longest bivalent in the heterogametic genotype, in agreement with the findings of Foresti *et al.* (1993). The relatively high incidence of unpaired segments in this terminal region observed in 6 of the 10 heterogametic males examined (25% of the nuclei examined), together with the much lower incidence of unpairing observed in the homogametic genotype in this study (2.6% of the nuclei examined, non-terminal) suggests the presence of a higher degree of non-homology in the heterogametic genotype than in the homogametic genotypes between the parental chromosomes forming the longest bivalent.

A similar cytogenetic analysis on XY wild type males also revealed similar levels of unpairing in the terminal region of the longest chromosomal pair (Carrasco *et al.*, 1997, Appendix 10). In that study, unpaired axial elements in the longest bivalent were observed in 5 of 6 XY wild type males, with a frequency of unpairing of 25.7% of the 35 nuclei examined in these males. A number of structural anomalies (i.e. self-folding of the terminal region, interaction of the longest bivalent with other bivalents and differential staining) previously described in the sex chromosomes of other vertebrate groups were also observed in XY wild type males. In addition, the analysis of 20 whole-nuclear SC spreads collected from YY 'supermales' showed the absence of unpairing in the terminal region of the longest bivalent, similarly to what was found in XX^{MT} males in the present work. These observations strongly support the hypothesis that the

terminal region of the longest bivalent in *O. niloticus* is undergoing a process of sex-chromosome differentiation.

A general requirement in the process of sex chromosome differentiation is the restriction of recombination in the heterogametic genotype between the regions containing the sex-determining genes (Solari, 1994). It is now widely accepted that the SC helps in the conversion of crossovers into functional chiasmata which assist in the orderly disjunction of the bivalents (Loidl, 1994). Thus the observation of unpaired axial elements in the terminal region of the longest bivalent may signal a change in the pattern of recombination in this region of the heterogametic genotype. This would be in agreement to the establishment of an XX/XY mode of sex determination mechanism in this species, supporting the hypothesis that the main sex-determining locus in *O. niloticus* is distally located to the centromere (Mair *et al.*, 1991).

However, two of the present observations require further interpretation: the presence of full synapsis between the lateral elements of the longest bivalent in 75% of the cases examined in the heterogametic genotype, and the observation of unpairing in the subterminal region of the same bivalent in the homogametic genotype (2.6% of the cases examined). Chromosomal pairing behaviour is affected by a mechanism termed *axial equalisation* which is characteristic of the sex chromosomes of most avian species studied (Hogan *et al.*, 1992; Solari, 1992). The ZW pair of avian sex chromosomes at early pachytene consists of two unequal LEs partially synapsed. During mid-pachytene, the Z axis gradually shortens until it becomes equalised and pairs in full with the W chromosome by late pachytene. Thus it is possible that the fully-paired lateral elements observed in the longest

bivalent in the heterogametic genotype represent non-homologous associations of the terminal region of the sex chromosomes of *O. niloticus* which would increase the stability of this bivalent by the end of pachytene. Regions of unpairing were also observed in the longest bivalent of the homogametic genotype, but both their low frequency and localisation (subterminal as opposed to terminal in the heterogametic genotype) suggest that rather than signalling regions of non-homology, they are a consequence of the delay in the completion of the synaptic process resulting from the length of this bivalent (Solari, 1994).

The pattern of synapsis observed in the meiotic behaviour of the longest bivalent in the present study seem to indicate the development of some degree of sex-chromosome differentiation of this chromosomal pair in the Nile tilapia. It should be noted that chromosomal heteromorphism is generally a late consequence of the establishment of a stable genetic sex-determining mechanism or 'master switch', in which a change in the recombination patterns between the heterogametic genotypes is the most likely primary event (Solari, 1994). In comparison with vertebrate species in which well-differentiated heteromorphic chromosomes are present, the Nile tilapia appears to be at a primitive stage in the process of sex-chromosome differentiation.

A final interesting observation in diploid genotypes is that, in spite of the lower mean gonadal weights and gonado-somatic indexes observed in XX^{MT} males, nuclei at the pachytene stage of meiosis were detected earlier (5.1 months p.f.) in XX^{MT} males than in heterogametic males (7.3 months p.f. in XY and 8.3 months p.f. in XY^{MT} males). This may represent an early commitment in genetically female

spermatogonia to enter meiosis, which would seem to accommodate the current views on the chronology of the early gonadal differentiation in *O. niloticus* (Alvencia-Casasuay and Cariño, 1988). The totality of the spreads analysed in diploid genotypes show almost all SCs having completed the process of synapsis. Thus, in agreement with previous observations on SC formation in the rainbow trout, a classical leptotene with complete but still unsynapsed AEs does not appear to exist in *O. niloticus* (Von Wettstein *et al.*, 1984; Oliveira *et al.*, 1995).

6.5.4 Chromosomal pairing in triploids

The observations recorded in this work on the nature of pairing in triploid genotypes deserve careful consideration. The presence of nuclei at the pachytene stage of meiosis was delayed in triploid genotypes compared to diploid genotypes (average male age of 6.65 months p.f. in diploids and 7.96 months p.f. in triploids). As observed in diploid genotypes, the first group in which nuclei at the pachytene stage of meiosis were detected was the XXX^{MT} group (at 5.3 months p.f.), which also had the lowest mean gonadal weight values (0.17 g) and gonado-somatic indices (0.31%) among the three triploid groups examined. The same age order observed in diploid genotypes was repeated in triploid groups, with nuclei at the pachytene stage of meiosis observed in the XXY group at 8.4 months p.f. and in the XXY^{MT} at 10.4 months p.f. This may represent, as noted for diploid genotypes, an earlier commitment in genetically female spermatocytes to enter the first meiotic prophase, and perhaps a delaying effect of methyltestosterone on the synaptic process in the XXY genotype.

The differences observed in TAEI length are difficult to interpret, since as noted for diploid genotypes, variations in TAEI are common among vertebrates and may indicate the attainment of different pachytene sub-stages at the moment of examination. Assuming that longer TAEI values represent earlier pachytene stages, the most advanced group examined was the XXX^{MT} genotype (with a mean TAEI value of 405.6 μm), followed by the XXY^{MT} genotype (TAEI= 471.1 μm) and the XXY genotype representing the earliest pachytene sub-stage (TAEI= 543.3 μm). The small sample size in the XXY group ($n= 3$), however, limited the sensitivity of the statistical

analysis to detect significant differences in mean TAEI values between the XXY and the XXY^{MT} genotypes, making a reliable assessment of the pattern of synapsis in this group difficult.

A helpful indicator for pachytene sub-staging may be the ratio of mean TAEI values between triploid and diploid genotypes. TAEI ratios are 1.53 for the XXX^{MT}/XX^{MT} , 2.18 for the XXY/XY and 1.35 for the XXY^{MT}/XY^{MT} genotypes. The XXX^{MT} and XX^{MT} groups seem thus to be at a very similar stage of pachytene, indicated by a ratio which is very close to the expected TAEI ratio between triploid and diploid nuclei (1.5). Considering that the XX^{MT} group are probably at a late pachytene sub-stage (which can be inferred from the lowest mean TAEI values and highest pairing extent among the diploid genotypes observed in this group), it seems reasonable to assume that the males in the XXX^{MT} group are also at a late pachytene sub-stage. TAEI ratios between triploid and diploid genotypes are also in good agreement with the previous interpretation that males in the XY^{MT} group are at an early sub-stage of pachytene (thus displacing the ratio XXY^{MT}/XY^{MT} below 1.5), and equally suggest that the XXY group are at an early sub-stage of pachytene (resulting in a displacement of the ratio XXY/XY above 1.5).

Taking into account the absolute TAEI values and the triploid/diploid ratios, a plausible interpretation of the data from this study would consider the XXY group to be at an early pachytene sub-stage, and the XXY^{MT} and XXX^{MT} at later sub-stages of pachytene. No comparative assessment on the pachytene sub-stage of the XXY^{MT} group with relation to the XXX^{MT} group is possible in this analysis: the higher TAEI values observed in XXY^{MT} males may indicate an earlier pachytene stage compared to the XXX^{MT} group; they may represent a genuine larger size of the SCs or they may originate from a superimposition of both factors in this group. Interestingly, the

finding of shorter TAEI values in homogametic genotypes in the present study resembles the situation described for diploid rainbow trout oocytes (Oliveira *et al.*, 1995) and some insects such as *Ephesia kuehniella* (Von Wettstein *et al.*, 1984), and differs from other vertebrates such as *Xenopus laevis* (Loidl and Schweizer, 1992) and humans (Wallace and Hulten, 1985) in which oocyte SCs have been found to be twice as long as spermatocyte SCs.

A different parameter considered in this study is the extent of pairing observed in triploid genotypes. Although lower in the XXY group, the extent of pairing did not significantly differ in the three groups examined (59% for the XXX^{MT} and XXY^{MT} groups, 56% for the XXY group) and it was close to the theoretical maximum extent of pairing in triploids (66%), excluding triploid pairing which was negligible (<0.3%) in all the groups examined. Again, the low sample size in the XXY group may have prevented the identification of a significantly lower extent of pairing in this group, but the high extent of pairing observed in all groups (89.4% of the theoretical maximum in MT-treated groups and 84.8% in the XXY group) suggests that a mechanism to ensure the maximum extent of pairing operates from an early stage in the process of synapsis in all groups examined. This would be in agreement with the process of synaptic adjustment previously described for other triploids (Loidl and Jones 1986; Thomas and Thomas, 1994; Oliveira *et al.*, 1995; Santos *et al.*, 1995).

The observed discrepancy in number of bivalents between the three groups analysed deserves special attention. It is important to note that the mean number of bivalents computed in this study probably represent the minimum number of

bivalents detectable in every nucleus, since the classification criteria used were very restrictive and biased towards preferentially scoring fully-paired bivalents rather than partially-paired ones, which were more difficult to trace. It seems obvious that pre-meiotic endoreduplication to hexaploid values (as described in *Poeciliopsis*), although possible, is a rare occurrence observed only in 1 of the 56 triploid nuclei examined, and does not represent the normal pairing pattern in triploid *O. niloticus*. An accurate assessment of the nature of pairing in triploid *O. niloticus* would require the direct observation of chiasmata; nevertheless the presence of a number of bivalents 2.6 times higher in the XXY^{MT} than in the XXX^{MT} group provides a strong indication for a mechanism of pairing correction to be operating in XXY^{MT} males.

The observation of a lower number of bivalents in XXY males (at an early sub-stage of pachytene) compared to XXY^{MT} males (at a late sub-stage of pachytene), with both groups showing a similar degree of pairing, suggests that the number of multivalents is reduced in favour of bivalents by late stages of pachytene in XXY^{MT} males. The corrective mechanism seems thus to modify the pairing pattern in heterogametic genotypes from a more random association of the AEs in early pachytene (as seen in XXY males with a mean bivalent number of 4.7) to a preferential II+I mode of pairing later in pachytene, indicated in XXY^{MT} males by a mean bivalent number of 9.8, with some males close to the maximum possible number of 22 bivalents (i.e. 16 bivalents observed in XXY^{MT} male number 3, Figure 6.23). This mechanism of pairing correction does not seem to operate in XXX^{MT} males, with a mean number of 3.7 bivalents per nucleus, and a maximum number of bivalents of 8 (XXX^{MT} neomale number 3, Fig. 6.23).

It is difficult to speculate on the significance of the positive correlation observed between mean TAEI values and the number of bivalents in triploid genotypes, because the present knowledge on meiotic pairing and recombination is limited, and because previous information remains inconclusive. There are several reports in the literature providing evidence for a correlated variation between mean TAEI values at pachytene and mean chiasma frequency at diplotene-metaphase I. Most of these emerge from analyses of sex-related differences in SC length and chiasma or crossover frequencies (Bojko, 1985; Wallace and Hulten, 1985; Jones and Croft, 1989), or from analysing the effects of additional heterochromatin in meiocytes, such as B chromosomes (Jones *et al.*, 1989) or heterochromatic knobs (Mogensen, 1977). A few of them, however, show correlated variation between both parameters within different families, unrelated to sex differences (Fox, 1973; Quevedo *et al.*, 1997).

In these studies, longer SCs are generally associated with higher chiasma/crossover frequencies. Similarly, organisms with low DNA/SC ratios generally have high recombination frequencies (Loidl, 1994), as exemplified by the correlation between the less dense DNA packaging and increased rate of recombination displayed by human-derived yeast artificial chromosomes compared with human DNA in its natural environment (Loidl *et al.*, 1995).

Considering that the 1C DNA (haploid content) of *O. niloticus* is 0.95 pg/cell (Majumdar and McAndrew, 1986), mean packing density is 7.2×10^3 pg of DNA (of one chromatid) per micrometer of SC in the Nile tilapia, according to the mean SC length calculated in this study (Table 6.3). Information on mean packing densities is scarce; however this value is lower than the mean packing density reported for the

rainbow trout (9.5×10^3 pg of DNA/ μm , Oliveira *et al.*, 1995), and the value observed in man (11.7×10^3 pg/ μm). It is however higher than the mean packing density calculated for the domestic fowl (5.8×10^3 pg/ μm) and for *Xenopus laevis* males (5.5×10^3 pg of DNA/ μm , Loidl and Schweizer, 1992), the lowest value reported among vertebrates. If the same relationship between TAEI or packing densities and recombination frequency was valid for *O. niloticus*, the longer SCs observed in XXY^{MT} males should correspond with a higher number of chiasmata, which might in turn make the operation of the suggested correcting mechanism in this group in resolving trivalents/multivalents with multiple chiasmata very difficult.

In the absence of detailed information on sex-specific recombination rates, the following model is proposed to explain the pairing pattern observed in triploid *O. niloticus* in this study. Triploid nuclei in *O. niloticus* enter the first sub-stage of pachytene meiosis, and by this time a process of synaptic adjustment maximises pairing and results in extensive, non-homologous synapsis with the probable occurrence of crossing-over involving both homologous and non-homologous chromosomes. This would be the situation observed in the XXY group.

Later in pachytene, a mechanism for pairing correction operates to rescue trivalents/multivalents and results in the preferential formation of bivalents and univalents. This mechanism transforms crossovers between homologous strands into functional chiasmata, resolves non-homologous crossovers and is somehow favoured by the presence of longer SCs. Perhaps the enzymatic machinery of recombination, known to be large protein complexes travelling along the DNA

strands, benefits from a lower packing density in the resolution of the crossovers. This is what is observed in XXY^{MT} males, where higher TAEI values may have facilitated the operation of the recombination machinery in resolving the crossovers in multivalents, driving the pairing pattern towards a II+I preferential mode of association. The subsequent normal (1:1) segregation of bivalents and a random segregation of the third set of univalents allows for the resolution of chromosomal disjunction in meiotic prophase I, and results in the production of aneuploid spermatozoa with ploidy levels which are between the haploid and the diploid values, as observed in other teleosts (Benfey *et al.*, 1986).

In homogametic genotypes, the operation of the mechanism for pairing correction is impaired, even despite possibly lower recombination rates than in heterogametic genotypes, and related perhaps to physical constraints for the enzymatic machinery of recombination to resolve the crossovers. The SCs in homogametic males may be inherently shorter than in normal heterogametic males, a situation already described for such genotypes in diploid rainbow trout oocytes (Oliveira *et al.*, 1995). The mechanism for pairing correction cannot resolve the crossovers in females, the chromosomes remain entangled as a result of crossovers between homologous (and possibly non-homologous) DNA strands, and the cell is prevented from entering the next meiotic division, causing an early arrest of gametogenesis in triploid females.

Although only a tentative model, it provides an explanation for the sex differences observed in the gametogenic progress in several species of triploid teleosts (Swarup, 1959b; Lincoln, 1981; Wolters *et al.*, 1982; Benfey and Sutterlin, 1984; Lincoln and Scott, 1984; Nakamura *et al.*, 1987, 1993; Piferrer *et al.*, 1994; Krisfalusi

and Cloud, 1997), and accomodates the data on structural differences (TAEL values) and pairing pattern (mean number of bivalents) observed between homogametic and heterogametic genotypes in this study. It would also provide some basis for the observation of some triploid rainbow trout males progressing further than others through spermatogenesis, as described in Chapter 5. These males may contain spermatocytes with larger SCs, which in turn may (or may not) be related to inter-individual variations in the levels of endogenous testosterone. Residual amounts of triploid or near-triploid spermatozoa could also originate in some males from the divisional reduction of hexaploid spermatogonia, as observed in the present work. On fertilisation, these may generate low numbers of aneuploid hypertriploid offspring, providing a possible explanation for the hypertriploid offspring observed in Chapter 5. Improvements in the techniques designed to obtain SC spreads in tilapia oocytes and the collection of sex-specific data on recombination rates in this and other species of teleosts will be of great help for assessing the accuracy of the model.

CHAPTER VII

GENERAL DISCUSSION

Triploidy induction results in an increase of cellular volumes and possibly, a decrease in cell numbers. In a cytomorphometric analysis conducted on Atlantic and coho salmon, the cell size of erythrocytes, leukocytes, brain and retinal cells was larger in artificially-produced triploids than in diploids (Small and Benfey, 1987). The authors inferred that since organ and body size were the same, cell numbers were proportionally reduced to cell size in triploids. Strüssman and Takashima found larger hepatocyte size in triploid pejerrey compared to diploids (1990). Swarup (1959b) reported a lower number of cells in the pronephric ducts of triploid *G. aculeatus* compared to diploids.

Concomitant with the increase in cell size resulting from triploidy induction and the subsequent alteration in volume: surface cellular ratios, the physiology, metabolism and behaviour of triploid individuals is also altered. Larger red blood cells of triploid fish transfer oxygen at lower rates than diploids, possibly because of their lower surface area:volume ratio (Holland, 1970). Graham *et al.* (1985) found reduced loading of oxygen on haemoglobin, lowered blood haemoglobin concentrations and a 68% reduction in maximum blood content in triploid Atlantic salmon relative to diploids. Triploidy decreases the aerobic swimming capacity of rainbow trout (Virtanen *et al.*, 1990). Protein energy catabolism and oxygen consumption was lower in triploid rainbow trout compared to diploids (Oliva-Teles and Kaushik, 1987). Phosphorylase and acid- α -glucosidase activities in hepatocytes differed between diploid and triploid masu salmon (Konishi *et al.*, 1991).

Triploids exhibited less aggressive behaviour than diploid fighting fish (Kavumparath and Pandian, 1992). Triploid ayu have lower sensitivity to sound and light than diploids (Aliah *et al.*, 1990). Triploid salamander larvae learn less quickly than diploids how to negotiate a simple maze (Fankhauser, 1955). Triploid grass carp do not grow as well as diploids when reared in direct competition with diploids for a limited amount of food, but do grow well when reared in all-triploid groups (Cassani and Caton, 1986). Results on growth performance of triploids must thus take into account the alteration in the metabolism and physiology brought about by triploidy induction, since digestive mechanisms or assimilation efficiencies may not be necessarily identical between triploid and diploid forms.

From a commercial perspective, the increase in carcass weight observed in triploids at 20 and 44 months of age in this study constitutes a valuable observation, illustrating the growth potential of triploid rainbow trout under a controlled culture environment. However, the optimisation of feeding regimes, taking into account the possibly different energetic requirements for artificial triploids, may result in further reduction in production costs and thus, more efficient production schemes. Wiley and Wike (1986) found a 10% decrease in ingestion rates for triploid grass carp compared to diploids. Special attention should thus be devoted to the preparation of rations and level of feeding when farming triploid teleosts, since much remains unknown about their energetic demands.

While the results from Chapter III on gonadal weights and gonado-somatic indices clearly illustrate the different impact of triploidy on male and female rainbow trout, Chapter IV provides new information on the pattern of gametogenesis in triploid rainbow trout. The findings on male gametogenesis are in general agreement with previous investigations (Lincoln and Scott, 1984; Nakamura *et al.*, 1987; Kobayashi *et al.*, 1993); however, the early blockage in oogenesis and the progressive masculinisation observed in triploid female gonads at late stages of their life cycle warrant further investigation. Similar to what has been reported in triploid female poultry (Thorne *et al.*, 1988; Fitzgerald and Cardona, 1993; Lin *et al.*, 1995a, b), triploid female trout seem mostly unable to differentiate a follicle. The suggestion discussed in Chapter IV of a possible alteration in somatic-to-germ-cells interactions resulting in the disruption of folliculogenesis in triploid females merits elucidation. The alteration in surface-volume ratios caused by triploidy induction may affect the number, distribution, structure and/or functionality of receptors in the cellular membrane of oocytes, granulosa or thecal cells, disrupting the intimate cell-to-cell communication events characteristic of folliculogenesis in vertebrates (Peters, 1978; Beers and Dekel, 1981; Browder, 1985; Guraya, 1986; Tyler and Lancaster, 1993; Moley and Schreiber, 1995).

It is difficult to speculate on the origin of the gonadal hermaphroditism observed in triploid females, since much remains unknown about the dynamics of sex differentiation in non-mammalian vertebrates. While the observation of gonadal hermaphrodites in homogametic (presumptive XXX) triploid female trout is somewhat puzzling, the appearance of spermatogenic areas in the gonads of

genetically-female triploids may be more easily explained in triploid heterogametic (ZZW) chickens, which are likely to contain in their chromosomal constitution the gene(s) responsible for male and female sexual differentiation (Thorne *et al.*, 1988; Fitzgerald and Cardona, 1993; Solari, 1994). Allen and Downing (1990) also report a significantly higher number of gonadal hermaphrodites during the gametogenesis of chemically-induced triploid oysters, possibly at the expense of triploid females. In the mouse, gonadal somatic cells are autonomously committed to differentiate in the male direction, independently of their genetic constitution; specific somatic-germ cells interactions are required for the normal differentiation of female somatic cells (Burgoyne *et al.*, 1988; Hashimoto *et al.*, 1990). Lately, new evidence obtained by studying the temperature-dependent process of sex determination in reptilians suggests that the male sex may be the default state of differentiation in these animals, and that the female condition must be imposed upon it (Merchant-Larios *et al.*, 1997). Perhaps male differentiation is also the default state in the rainbow trout, thus explaining the appearance of gonadal hermaphrodites after the arrest of the oogonia observed in triploid females. Complex as it may be, the study of the dynamics and genetic basis of sex differentiation in non-mammalian vertebrates looks increasingly fascinating.

The study of the reproductive potential of triploid rainbow trout males described in Chapter V provides valuable evidence for the generation, although in very limited numbers, of viable offspring by triploid trout males when crossed to normal (diploid) females. Limited fertility of triploid teleosts may not be exclusive

to males; some triploid female salmonids (Benfey, 1995) and non-salmonid teleosts (Cimino, 1972; Penman *et al.*, 1987; Brämick *et al.*, 1995) have been shown to develop large gonads containing seemingly mature ova. Thus triploidy may greatly reduce the possibility of genetic interaction between genetically manipulated, farmed (transgenic or not) and natural populations, but caution should be exercised when recommending triploidy induction for the reproductive containment of teleost populations, since the manipulation cannot totally prevent the production of gametes. Comprehensive analyses of the reproductive potential of artificially-induced triploids in different teleost species (and aquatic organisms in general) are needed to further assess the reliability of the technique.

The use of innovative cytogenetic techniques (i.e. synaptonemal complex analysis; Moses, 1956; Loidl, 1994; Egel, 1995; Hasenkampf, 1996) appears very promising for improving our understanding of the genetics of sex determination in many teleost species, as exemplified by the findings presented in Chapter VI on the cytological basis for sex determination in the Nile tilapia. These techniques have also proven to be of great value in the meiotic analysis of polyploid plants and fungi (Rasmussen *et al.*, 1981; Sherman *et al.*, 1989; Vincent and Jones, 1993; Thomas and Thomas, 1994). The application of the analysis of the synaptonemal complex in polyploid teleosts should thus become an essential tool for improving our understanding of the alterations of the meiotic process brought about by triploidy induction in these animals.

The observation of a different pattern of chromosomal pairing during the meiosis of male and female autotriploid Nile tilapia described in Chapter VI deserves special consideration. Meiotic arrest in female triploid tilapia seems to be

independent of phenotype; in other words, the inability of genetically female spermatocytes to successfully progress through chromosomal disjunction bears no relation to the disruption of folliculogenesis observed in triploid ovaries. It thus seems reasonable to interpret the failure of the mechanism for pairing correction observed in triploid females as the *primary* cause of meiotic arrest in triploid female gametogenesis; the disruption of folliculogenesis observed in triploid ovaries would thus be subsequent to the meiotic arrest of nested triploid oogonia. Oyster eggs, and those of molluscs in general, do not have to complete any meiotic divisions before oocyte growth (Masui, 1985; Allen and Downing, 1990; John, 1990). This may explain the considerable development of female gametes observed in artificially-produced triploid oysters and other molluscs (Allen *et al.*, 1986, 1990). In these species, the alteration in cellular dimensions caused by triploidy induction may result in the disruption of folliculogenesis, thus driving the differentiation of somatic cells into the male direction, which would explain the observation of high numbers of hermaphroditic individuals among triploid populations of oysters (Allen *et al.*, 1990). Oogonia in the rainbow trout enter the first meiotic division at a much earlier stage in the process of folliculogenesis than oogonia in molluscs (stages 1-2 of primary oocyte growth; Masui, 1985; Bromage and Cumaranatunga, 1988; Tyler and Sumpter, 1996). Thus in the rainbow trout, and triploid finfish in general, the main cause of the disruption in oogenesis would be the meiotic failure resulting from the nature of pairing in genetically female primary oocytes; the disruption in folliculogenesis would be the consequence rather than the cause of oogonia/primary oocyte arrest in triploid ovaries.

Nevertheless, the observation of low numbers of ova developing to maturity in several triploid finfish requires further explanation. It is possible to speculate, in a similar way to that suggested in Chapter VI to explain the observation of some triploid males progressing further in meiosis, that oocytes with longer synaptonemal complexes or lower DNA packing ratios may be able to overcome the difficulties during the resolution of crossovers in prophase I, escaping the meiotic blockage and further advancing in folliculogenesis and oocyte growth. Longer SCs or lower packing ratios may represent a random occurrence resulting from inter-cellular variations present within the oocyte pool, or perhaps sustained levels of endogenous testosterone over time, which is present at reduced (but not significantly so) levels in triploid female rainbow trout (Nakamura and Nagahama, 1987) may result in longer SC lengths and thus facilitate the resolution of the crossovers. Much remains to be known about the somatic-to-germ cells interactions in the developing gonad; in view of the present information these explanations remain merely speculative.

Triploidy is a lethal condition in mammals, with most triploid mammalian embryos dying at some stage between implantation and term (Kaufman, 1991; Henery and Kaufman, 1993). On the contrary, triploid fish are easily produced and their overall viability does not appear to be seriously compromised (Swarup, 1959; Cuéllar and Uyeno, 1972; Chourrout, 1980; Hussain, 1996). The induction of triploidy can be easily accomplished in most commercially-important aquatic species, and results in a severe limitation of their reproductive potential.

Taking into account the results from the present Thesis on the genotype-related differences observed in chromosomal pairing pattern during meiosis, it seems clear that further progress in our understanding of the basic mechanisms involved in the reproductive blockage observed in triploid teleosts will open new possibilities for the generation of sterile fish (and aquatic organisms in general) stocks.

In addition to the practical applications of triploidy induction in the prevention of sexual maturation in cultivated teleost species, triploid fish stand as a very promising model for the study of the effects of multiple gene dosage on gene expression and gene regulation in polyploid vertebrates.

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APPENDICES

APPENDIX 1

Isoton II composition:

Sodium chloride	7.9 g/L of water
Sodium hydrogen orthophosphate	1.9 g/L of water
EDTA, disodium salt	0.4 g/L of water
Potassium chloride	0.4 g/L of water
Sodium dihydrogen orthophosphate	0.2 g/L of water
Sodium fluoride	0.3 g/L of water

(Filtered to 2 microns)

APPENDIX 2

10% Phosphate-buffered Formalin composition:

Formaldehyde 40%	100ml
Distilled water	900 ml
$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	4g
Na_2HPO_4	6g
NaCl	4g

APPENDIX 3

Processing schedule for paraffin embedding:

50% methylated spirits	1 hr
80% methylated spirits	2 hr
100% methylated spirits	2 hr
100% methylated spirits	2 hr
100% methylated spirits	2 hr
100% ethanol	2 hr
Chloroform	2 hr
Chloroform	1 hr
Chloroform	1 hr

First infiltration in paraffin wax at 60°C for 2 hr

Second infiltration in paraffin wax at 60°C for 2 hr

APPENDIX 4

Haematoxylin-eosin staining protocol (modified from Carleton, 1980):

Xylene	5 min
100% Ethanol	2 min
Methylated spirits	1.5 min
Tap water wash	0.5 min
Haematoxylin	5 min
Tap water wash	0.5 min
1% acid alcohol	4 quick dips
Tap water wash	1 min
Scott's tap water substitute	0.5 min
Tap water wash	1 min
Eosin	2 to 3 min
Tap water wash	0.5 min
Methylated spirits	1 min
100% Ethanol	2 min
100% Ethanol	1.5 min
Xylene	5 min
Xylene	5 min
Mount	

APPENDIX 5

Staining protocol for the periodic acid-Schiff reaction counterstained with Heidenhain's iron haematoxylin stain (modified from Carleton, 1980):

Preparation of the reagents:

(a) The iron alum mordant

Ferric ammonium sulphate	5 g
Distilled water	100 ml

(b) The iron alum solution

Ferric ammonium sulphate	2.5 g
Distilled water	100 ml

(c) The haematoxylin bath

Haematoxylin	0.5 g
100% Ethanol	10 ml
Distilled water	90 ml

Staining protocol:

Tap water wash	1 min
1% aqueous periodic acid	5 min
Tap water wash	5 min
Distilled water	0.5 min
Schiff's reagent	15 min
Tap water wash	30 min
Iron alum mordant	30 min
Distilled water	1 min
Haematoxylin	30 min
Tap water wash	5 min
Iron alum solution	10-20 min

Differentiate in the iron alum solution, controlling microscopically by removing the slide from the alum solution and washing briefly in tap water to halt the process during examination. Continue differentiation and examination until sections are suitably de-stained, noting that red blood cells take up and retain the stain strongly.

Tap water wash	10 min
90% Ethanol	2 min
100% Ethanol	2 min
100% Ethanol	1.5 min
Xylene	5 min
Xylene	5 min
Mount	

APPENDIX 6

Staining protocol for the Masson's trichrome stain (modified from Carleton, 1980).

Preparation of solutions:

Cytoplasmic (plasma) stain:

1% Ponceau de Xylidine in 1% acetic acid	2 parts
1% Acid fuchsin in 1% acetic acid	1 part

Differentiator and mordant:

1% phosphomolybdic acid in distilled water

Fibre stain:

2% Light green in 1% acetic acid

Weigert's iron haematoxylin:

Solution A (stain):

Haematoxylin	1 g
Absolute alcohol	100 cm ³

Solution B (mordant):

30% aqueous ferric chloride (anhydride)	4 cm ³
Concentrated hydrochloric acid	1 cm ³
Distilled water	95 cm ³

Solutions A and B are stored separately and mixed immediately before use to 1:1 proportions.

(Appendix 6)

Protocol:

Weigert's iron haematoxylin	10 min
Wash well in distilled water	
0.5% HCl in 70% ethanol	0.5-1 min
Wash well in distilled water	
Cytoplasmic stain	6 min
Wash well in distilled water	
Differentiator	0.5-1 min
Wash well in distilled water	
Fibre stain	2-5 min
1% Acetic acid	1.5 min
100% Alcohol	0.5 min
Xylene	
Mount	

APPENDIX 7

Average relative frequencies of cell types observed in diploid and triploid ovaries at different age stages.

Values indicate means \pm standard deviations. Significantly different means ($P < 0.05$) appear underlined.

Key:

O, oogonia;

1-7, oocyte stages (after Bromage and Cumaranatunga, 1988);

ATR, atretic oocytes;

SPT, spermatogenic cells;

POF, post-ovulatory follicles;

STR, stroma;

G.E., germinal epithelium;

G.S., glandular structures;

NCR, necrosis;

INF, inflammatory cells;

MYE, myelocytes.

(Appendix 7)

AGE	7 MONTHS		15 MONTHS		20 MONTHS		25 MONTHS		44 MONTHS	
PLOIDY (N)	2n (11)	3n (11)	2n (8)	3n (9)	2n (6)	3n (6)	2n (10)	3n (10)	2n (5)	3n (10)
0	3.20±4.31	71.14±10.13	0.00±0.00	57.32±10.7	0.00±0.00	61.42±6.78	0.00±0.00	43.43±11.13	0.00±0.00	30.37±17.33
1	1.16±2.22	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
2	10.56±3.30	0.00±0.00	5.21±3.94	0.00±0.00	0.07±0.12	0.00±0.00	0.11±0.15	0.00±0.00	0.00±0.00	0.12±0.38
3	64.84±5.93	0.00±0.00	32.73±14.0	0.00±0.00	4.17±2.13	0.00±0.00	7.88±1.84	0.11±0.36	1.37±1.30	1.26±3.34
4	0.99±2.21	0.00±0.00	41.68±16.6	0.00±0.00	0.72±0.70	1.49±3.66	9.59±4.28	1.92±3.81	0.69±0.94	3.07±3.15
5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	9.18±22.43	0.00±0.00	7.62±4.28	0.00±0.00	1.20±2.23	1.59±3.09
6	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	50.18±24.84	0.00±0.00	0.00±0.00	0.00±0.00	28.88±14.9	0.00±0.00
7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
ATR	2.76±3.66	0.11±0.35	2.40±1.34	0.00±0.00	5.43±2.76	0.00±0.00	9.21±3.77	0.00±0.00	31.39±5.60	0.00±0.00
SPT	0.00±0.00	0.22±0.70	0.00±0.00	0.96±2.23	0.00±0.00	1.67±2.79	0.00±0.00	4.48±4.93	0.00±0.00	12.21±13.17
POF	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	34.63±10.92	0.00±0.00	9.79±11.37	0.00±0.00
STR	3.22±5.42	0.44±1.41	7.39±2.56	0.38±1.15	2.20±1.74	1.85±4.54	6.61±5.09	1.42±2.61	7.87±4.41	0.00±0.00
G.E.	0.31±0.75	0.34±0.77	0.00±0.00	3.94±2.34	0.00±0.00	3.34±3.92	0.00±0.00	7.02±4.96	0.00±0.00	4.39±3.95
G.S.	0.00±0.00	2.04±4.46	0.00±0.00	1.42±2.62	0.00±0.00	0.00±0.00	0.00±0.00	6.28±10.18	0.00±0.00	3.67±10.48
FIBRES	0.00±0.00	9.27±10.24	0.00±0.00	18.41±6.86	0.00±0.00	13.70±5.14	0.00±0.00	13.36±10.12	0.00±0.00	16.84±9.49
NCR	0.00±0.00	5.81±8.76	0.00±0.00	5.57±5.68	0.05±0.12	1.88±3.08	0.00±0.00	3.24±5.01	0.00±0.00	11.99±12.27
INF	0.00±0.00	1.86±3.61	0.00±0.00	1.38±2.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
MYE	0.00±0.00	0.00±0.00	0.05±0.14	0.06±0.19	0.16±0.26	1.85±2.30	0.29±0.51	1.45±2.51	0.00±0.00	1.15±1.44

APPENDIX 8

Average relative frequencies of cell types observed in diploid and triploid testes at different age stages.

Values indicate means \pm standard deviations. Significantly different means ($P < 0.05$) appear underlined.

Key:

GON A= type A spermatogonia;

GON B= type B spermatogonia;

SPC 1= primary spermatocytes;

SPC 2= secondary spermatocytes;

SPT= spermatids;

SPZ= spermatozoa;

INT= interstitial cells;

SER= Sertoli cells;

STR, stroma;

NCR= necrosis.

(after Billard, 1983; 1986; 1990).

(Appendix 8)

AGE	7 MONTHS		15 MONTHS		20 MONTHS		25 MONTHS		44 MONTHS	
	2n (11)	3n (9)	2n (13)	3n (11)	2n (4)	3n (4)	2n (9)	3n (10)	2n (1)	3n (1)
GON A	22.43±14.21	26.33±9.90	15.10±13.8	2.97±5.03	0.00±0.00	0.00±0.00	0.07±0.19	0.00±0.00	0.00	0.00
GON B	43.50±18.40	33.48±16.89	34.58±19.5	15.05±16.48	0.00±0.00	0.78±0.86	4.42±2.96	2.30±1.90	0.00	0.00
SPC 1	4.83±15.27	10.53±20.08	7.54±9.54	26.47±14.95	0.53±0.59	65.13±3.50	0.15±0.29	14.21±9.81	36.46	56.17
SPC 2	1.99±6.29	1.50±4.26	1.39±2.71	21.34±18.53	32.74±1.17	11.11±4.06	0.31±0.66	22.12±17.42	15.63	18.24
SPT	1.52±4.79	0.58±1.64	4.60±8.20	4.64±5.13	20.78±1.95	1.12±0.74	5.05±5.11	8.12±5.43	8.33	11.88
SPZ	0.19±0.60	0.00±0.00	15.42±28.2	6.92±13.02	39.88±3.03	0.00±0.00	68.49±6.28	31.77±22.63	33.33	4.86
INT	14.21±10.56	0.93±2.62	6.68±4.69	6.50±4.81	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00	0.00
SER	0.66±1.79	12.42±7.14	0.80±1.76	0.25±0.61	0.26±0.45	2.13±3.69	0.00±0.00	0.00±0.00	0.00	0.00
STR	7.47±6.98	8.38±4.39	2.37±2.26	3.32±3.13	2.34±1.33	13.80±1.38	12.85±3.42	11.80±4.30	5.21	1.56
NCR	0.00±0.00	0.69±1.39	0.21±0.42	0.13±0.27	0.26±0.29	3.25±2.59	0.21±0.42	1.11±1.99	0.00	3.99
FIBRES	2.46±3.42	4.69±7.23	7.72±5.01	6.00±7.58	0.26±0.45	0.00±0.00	0.89±2.52	0.14±0.42	0.00	0.35

APPENDIX 9

List of Communications

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CARRASCO, L., DOROSHOV, S., PENMAN, D., BROMAGE, N. 1997. Sexual differentiation and gametogenesis in triploid trout: the same story as in triploid chickens? *Proceedings of the First International Symposium on Vertebrate Sex Determination*, p 23. Honolulu, Hawaii, U.S.A. (Oral Communication).

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APPENDIX 10

Evidence for the presence of sex chromosomes in the Nile tilapia (*Oreochromis niloticus*) from synaptonemal complex analysis of XX, XY and YY genotypes

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ABSTRACT

A cytogenetic analysis of chromosome synapsis was carried out during the first meiotic prophase of the Nile tilapia, *Oreochromis niloticus*. Three different genotypes were studied: XX sex-reversed males, 'wild-type' (XY) males and YY 'supermales'. Transmission electron-microscope analysis of synaptonemal complex spreads revealed the presence of 22 fully paired bivalents during pachytene in both homogametic genotypes. In the heterogametic genotype, an incompletely paired segment was frequently observed during the process of meiotic synapsis in the terminal region of the longest bivalent. The presence of this unpaired segment, together with several features characteristic of sex-chromosome behaviour during meiosis, suggests the existence of a non-homologous region in this chromosomal pair in the heterogametic genotype, and provides cytological evidence for the chromosomal basis of sex determination in *O. niloticus*. The usefulness of synaptonemal complex analysis for the understanding of sex determination and its relevance in the management of species of aquacultural importance are discussed.

KEY WORDS: Cytogenetics, Meiosis, Sex Chromosomes, Synaptonemal Complex.

INTRODUCTION

Sex control in farmed fish stocks remains a critical objective for the aquaculture industry, since in most commercial species growth patterns and energy investment into reproduction differ between sexes. In the Nile tilapia (*Oreochromis niloticus*), one of the most important commercial species for food production world-wide, strategies for the generation of all-male populations are designed to combine the benefits of faster growth rates in males with the suppression of the problems posed by precocious sexual maturation, early breeding and competition from progeny, particularly under pond culture conditions.

The production of all-male populations has been successfully achieved by the oral administration of natural or synthetic androgens to masculinise sexually-undifferentiated fry (Tayamen and Shelton, 1978). Although this is a technique widely used today it presents a number of practical limitations, which combined with the increasing consumer resistance to the use of hormones in food production, limit its general applicability (Mair *et al.*, 1987). An alternative strategy in the production of monosex populations is the use of novel YY 'supermale' genotypes (Scott *et al.*, 1989; Myers *et al.*, 1995), which when crossed to 'normal' XX females should yield large numbers of all-male progeny. The genetic rationale underlying this approach is the presence of a Lygaeus type of sex determination in *O. niloticus* in which males are the heterogametic sex (Penman *et al.*, 1987). On the basis of the sex ratios observed in sex reversal, gynogenesis and triploidy experiments, a monofactorial model for sex determination has been hypothesised in which sex is predominantly determined by a single locus located distantly to the centromere, albeit possibly influenced by one or several secondary sex-modifying factors (Mair *et al.*, 1991). A number of constraints are also evident in this methodology: the initial phase in the generation of YY supermales is complex and time consuming, hormone treatment is still required for the feminisation of male genotypes and more importantly, some YY supermales do not yield the predicted all-male ratios (Mair *et al.*, 1991). It is thus clear that

further progress in the development of reliable techniques for sex control requires a better understanding of the sex determination mechanism operating in *O. niloticus*, with the ultimate goal of identifying and understanding the *modus operandi* of the sex-determining gene(s) present in this species.

A serious obstacle standing in the way is the absence of genetic markers for sex in *O. niloticus*. Avtalion *et al.* (1976) identified male-specific proteins in *O. niloticus* using polyacrylamide gel electrophoresis, but their use for the large-scale identification of sex is impaired by the complexity and cost of the technique, and they appear to be sex-limited rather than sex-linked (Mair *et al.*, 1991). Although Nijhar *et al.* (1983) reported the presence of a size heteromorphism in the longest chromosomal pair of *O. niloticus* females, suggesting the existence of a ZZ/ZW sex determination mechanism, later light microscopy-based studies failed to reveal sex-linked morphological differences in the karyotype of this species (Majumdar and McAndrew, 1986). A more powerful tool for karyotypic analysis is the electron-microscopical observation of the synaptonemal complex (SC), a protein scaffold which mediates in chromosomal pairing and recombination during the zygotene and pachytene stages of meiotic prophase I (Moses, 1956; Loidl, 1994; Egel, 1995; Hasenkampf, 1996). SC morphogenesis can be first visualised by the appearance of short stretches of protein cores called *axial elements* along each pair of sister chromatids. Chromosome synapsis proceeds by the establishment of intimate associations between homologous axial elements (usually near the telomeres), which once assembled into the SC are then referred to as *lateral elements*. The area between two lateral elements is called the *central region*, where densely staining, spheroidal structures called *recombination nodules* are observed (Von Wettstein *et al.*, 1984). These nodules are thought to be the cytological visualisation of large protein complexes involved in the enzymology of recombination, since their frequency and distribution correspond to the frequency and distribution of crossovers (Carpenter, 1988). After pachytene, the SC proteins begin to disassemble and

chiasmata are visible as sites of attachment between non-sister chromatids (Jones, 1987). Although the precise role of the SC in recombination is still the subject of ongoing research, the analysis of the SC stands as a useful tool to investigate abnormal pairing configurations associated with chromosomal differences between the parents, having been successfully used for the meiotic analysis of many plant and animal species, including teleosts (Foresti *et al.*, 1983; Wise and Nail, 1987; Lin and Yu, 1991; Oliveira *et al.*, 1995).

The analysis of SCs in *O. niloticus* wild-type males has revealed the occurrence of a size heteromorphism in the lateral elements of the largest bivalent, associated with the presence of an incompletely paired segment in the terminal region of this chromosomal pair (Foresti *et al.*, 1993). In this work we present the results of a cytogenetic analysis of chromosome synapsis designed to investigate the possible sex-linkage of such heteromorphism by studying the three different sexual genotypes of *O. niloticus*: XX, XY and YY.

MATERIALS AND METHODS

Experimental animals

O. niloticus individuals used in this study derive from a Lake Manzala (Egypt) population which has been extensively characterised by mitotic and meiotic karyology, electrophoresis and recombination rates studies (McAndrew and Majumdar, 1983; Majumdar and McAndrew, 1986; Mair *et al.*, 1991). All fish were maintained in warm water ($T = 27 \pm 2$ °C) recirculation systems under similar photoperiod (14L:10D), density and feeding regimes. In preliminary studies of SC spreads, oocytes were less amenable than spermatocytes to the spreading process and yolk interfered with the staining method. Consequently, it was decided to use sex-reversed males as a source of XX genotypes. An all-female population was produced by crossing XX sex-reversed males with wild-type females and progeny testing for all-female offspring. Six XX males (5 months of age, 30 g body weight, 12 cm fork length) were selected from a stock generated by oral administration of 17α -methyltestosterone (50 mg/kg of food during 30 days after first feeding) to this XX population. Six 'wild type' males of similar age, body weight and fork length to the XX sex-reversed males were selected on the basis of their gonadal appearance. Three progeny-tested YY 'supermales' were available for this study. One of these (4 years of age, 0.6 kg body weight, 40 cm fork length) was generated by androgenesis (Myers *et al.*, 1995). The other two (2 years of age, 250 g body weight, 30 cm fork length) were produced by crossing YY males to sex-reversed YY females and then progeny testing for all-male offspring.

Synaptonemal complex spreading

The preparation of SC specimens was carried out following the protocol of Foresti *et al.* (1993) with several minor modifications. After overanesthetising the males by immersion in a 0.01% benzocaine solution, the gonads were dissected, placed in a Petri dish containing 3 ml of Hank's saline solution (Sigma-Aldrich, Dorset, UK; adjusted to pH 8.5 with 1N NaOH) and carefully minced with two razor blades. The resulting cell suspension was aspirated with a Pasteur pipette and transferred into a sterile centrifuge tube placed in ice, where it was allowed to settle for 1 hr. The cell suspension, spreading medium (0.2% Lipsol[®] (LIP, Yorkshire, England) in distilled water adjusted to pH 8.5 with 0.01 M sodium tetraborate buffer) and a 0.2 M sucrose solution were added to treated histological slides in 1:2:2 proportions and gently mixed. Histological slides (Superfrost[®] BDH, Dorset, UK) had been thoroughly washed in soapy water, rinsed in distilled water, air dried and dipped in a 0.75% (w/v) solution of pioloform[®] plastic (Agar Scientific, Essex, UK) dissolved in reagent-grade chloroform. After 5 min exposure to the spreading medium, 1 ml of 4% paraformaldehyde fixative (buffered to pH 8.5 with sodium tetraborate) was added to each slide and they were then left to dry overnight in a vertical position in a fume cupboard. The slides were rinsed and air dried before staining.

Staining and preparation of specimens for electron microscopy (EM)

Plastic-coated slides bearing surface-spread nuclei were coverslipped with 250 μm nylon cloth mesh (Plastok Associates, Merseyside, UK) and stained with a 50% silver nitrate solution at 50 °C for 50 min (Kodama *et al.*, 1980). Suitably spread nuclei were identified under the light microscope (Olympus Optical Co., Japan) and their position was recorded on the plastic coating using a permanent marker. The plastic was scored with a sharp razor blade along the edges of the slide and floated off in distilled water. After 50-mesh EM copper grids (Agar Scientific, Essex, UK) were carefully positioned over the marks, a small piece of absorbent paper was dropped over the plastic film, and the

resulting sandwich was quickly picked up and left to dry with the plastic film facing up. After drying, the EM grids were detached from the plastic by scoring around their edge with a fine paintbrush dipped in chloroform. The grids were examined at 80 kV using a Philips 301 transmission EM.

RESULTS

In the 95 spermatocytes where complete nuclei were examined, SC spreads contained 22 silver-stained bivalents at the pachytene stage of meiotic prophase I, according to the staging criteria set by Villagómez (1993)(Fig 1a). Their lateral elements appeared as two well-differentiated parallel filaments spanning the bivalents from telomere to telomere, with a distinctly stained attachment plaque visible at both of their ends (Fig 1b). No clear structure was revealed by the silver-staining technique in the central region of the SCs, and the kinetochores, although evident in some cases, could not be consistently visualised in each bivalent. No obvious bouquet arrangement of the bivalents was observed. Equally, no broken lateral elements were observed, and chromosomal interlocking, although present in some cases, was rare (Fig 1c). One of the bivalents was conspicuously longer than the other 21 in all nuclei analysed, measuring approximately 16 μm in length (Fig 1d). The lateral elements of the 21 shorter bivalents paired in full along their length in all nuclei examined, presenting the normal SC morphology characteristic of other vertebrate groups. In the longest bivalent, however, the following anomalies were observed: partially unpaired axial elements, self-folding, interaction with other bivalents and differential staining. Unpairing of the axial elements affected the terminal region of the longest bivalent when it was observed in the heterogametic males, while it was restricted to the central and/or subterminal region of the longest bivalent in the two nuclei of the homogametic genotypes where unpairing was observed (Figs. 1e and 1f). Table 1 summarises the pairing patterns in the terminal region and structural anomalies observed in the longest bivalent by genotype. Unpaired axial elements in the terminal region of the longest bivalent were observed in 5 of the 6 heterogametic males examined (Figs. 2 a-c). Unpaired axial elements appeared of unequal length in 3 of the 9 cases recorded in wild-type males (Figs. 2c and 2d), while no obvious size heteromorphism was visible in the 2 cases observed in the homogametic genotypes. When no region of unpairing was visible in

the longest bivalent (97.5% of the cases examined in XX males, 74.3% in wild-type males and 95% in YY males) the lateral elements paired uniformly across its length (Fig 2e). The terminal region of the longest bivalent formed a loop folding on itself in one nucleus of a wild-type male (2.8%)(Fig 2f), while in another male of the same group the unpaired axial elements of this bivalent established partial synapses with three other bivalents forming a complex multivalent (Fig 2g). Staining intensity was noticeably darker and the lateral elements of the longest bivalent appeared slightly thickened in 2 of the nuclei examined in XX and wild-type males (5% and 5.7%, respectively)(Fig 2h).

DISCUSSION

The results from this work are in agreement with the mitotic karyotype reported by several authors for *O. niloticus* (Jalabert *et al.*, 1971; Arai and Koike, 1980; Nijhar *et al.*, 1983; Majumdar and McAndrew, 1986), with a diploid number of $2n=44$ corresponding to the 22 bivalents observed in this study. The number and morphology of the bivalents observed in this work are also in agreement with the previously reported SC karyotype of *O. niloticus* (Foresti *et al.*, 1993), although we could not consistently identify the kinetochores or chromosomes bearing nucleolar-organising regions (NORs), possibly as a result of technical limitations in our staining protocol. Similar features to the ones we observed in the longest bivalent (i.e. self-folding, interaction with other bivalents, differential staining) have been reported in the sex-chromosomes of other vertebrate species (Solari, 1994). Perhaps the most significant observation is the presence of unpaired segments in the terminal region of the longest bivalent in the heterogametic genotype, in agreement with the findings of Foresti *et al.* (1993). The relatively high incidence of unpaired segments in this terminal region observed in 5 of the 6 heterogametic males examined (25.7% of the nuclei examined), together with the much lower incidence of unpairing observed in the homogametic genotypes in this study (3.3% of the nuclei examined) suggests the presence of a higher degree of non-homology in the heterogametic genotype than in the homogametic genotypes between the parental chromosomes forming the longest bivalent. This observation supports the hypothesis that the terminal region of the longest bivalent in *O. niloticus* is undergoing a process of sex-chromosome differentiation. A general requirement in the process of sex chromosome differentiation is the restriction of recombination in the heterogametic genotype between the regions containing the sex-determining genes (Solari, 1994). It is now widely accepted that the SC helps in the conversion of crossovers into functional chiasmata which assist in the orderly disjunction of the bivalents (Loidl 1994). Thus the observation of unpaired axial elements

in the terminal region of the longest bivalent may signal a change in the pattern of recombination in this region of the heterogametic genotype. This would be in agreement to the establishment of an XX/XY mode of sex determination mechanism in this species, supporting the hypothesis that the main sex-determining locus in *O. niloticus* is distally located to the centromere (Mair *et al.*, 1991). However, two of the present observations require further interpretation: the presence of full synapsis between the lateral elements of the longest bivalent in 74.3% of the cases examined in the heterogametic genotype, and the observation of unpairing in the subterminal region of the same bivalent in the homogametic genotypes (3.3% of the cases examined). Chromosomal pairing behaviour is affected by a mechanism termed *axial equalisation* characteristic of the sex chromosomes of most avian species studied (Hogan *et al.*, 1992; Solari, 1992). The ZW pair of avian sex chromosomes at early pachytene consists of two unequal lateral elements partially synapsed. During mid-pachytene, the Z axis gradually shortens until it becomes equalised and pairs in full with the W chromosome by late pachytene. Thus it is possible that the fully-paired lateral elements observed in the longest bivalent in the heterogametic genotype represent non-homologous associations of the terminal region of the sex chromosomes of *O. niloticus* which would increase the stability of this bivalent by the end of pachytene. Our finding of a length heteromorphism in the unpaired axial elements of the longest bivalent in the heterogametic genotype (8.6% of the cases examined), which was not observed in other nuclei of the same individuals, provides further support for the presence of a mechanism of axial equalisation operating in *O. niloticus*. Regions of unpairing were also observed in the longest bivalent of the homogametic genotypes, but both their low frequency and localisation (subterminal as opposed to terminal in the heterogametic genotype) suggests that rather than signalling regions of non-homology, they are a consequence of the delay in the completion of the synaptic process resulting from the length of this bivalent.

Considered as a whole, the features observed in the meiotic behaviour of the longest bivalent seem to indicate the development of some degree of sex-chromosome differentiation of this chromosomal pair in the Nile tilapia. It should be noted that chromosomal heteromorphism is generally a late consequence of the establishment of a stable genetic sex-determining mechanism or 'master switch', in which a change in the recombination patterns between the heterogametic genotypes is the most likely primary event (Solari, 1994). In comparison with vertebrate species in which well-differentiated heteromorphic chromosomes are present, the Nile tilapia appears to be at a primitive stage in the process of sex-chromosome differentiation.

CONCLUSIONS

The investigation of chromosome synapsis during meiosis by the analysis of the synaptonemal complex has provided valuable information on the process of sex-chromosome differentiation taking place in the Nile tilapia. The observation of several features characteristic of sex-chromosomes in the longest bivalent (unpaired axial elements, self-folding and interaction with other bivalents in the heterogametic genotype) provides cytological evidence for the chromosomal basis of sex determination in *O. niloticus*. The combination of synaptonemal complex analysis with well-established methodologies for sex reversal offers new possibilities for the advancement in our understanding of the sex-determining mechanisms operating in aquaculturally-important species.

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TABLES

Table 1. Pairing patterns in the terminal region and number of structural anomalies observed in the longest bivalent during the pachytene stage of meiosis in *O. niloticus*.

Genotype	No. of nuclei analysed	Pairing pattern in terminal region		Structural anomalies		
		Unpaired AEs	Paired LEs	S	I	D
XX						
male 1	5	0	5	0	0	1
male 2	7	0	7	0	0	0
male 3	6	0	6	0	0	0
male 4	8	0	8	0	0	0
male 5	7	0	7	0	0	0
male 6	7	0	7	0	0	1
TOTAL	40	0	40 (100%)	0	0	2 (5%)
XY						
male 1	7	1	6	0	0	0
male 2	5	2	3	0	0	1
male 3	6	1	5	0	0	0
male 4	5	0	5	1	0	0
male 5	6	2	4	0	0	0
male 6	6	3	3	0	1	1
TOTAL	35	9 (25.7%)	26 (74.3%)	1 (2.8%)	1 (2.8%)	2 (5.7%)
YY						
male 1 (*)	7	0	7	0	0	0
male 2	7	0	7	0	0	0
male 3	6	0	6	0	0	0
TOTAL	20	0	20 (100%)	0	0	0

AEs, axial elements; LEs, lateral elements; S, self-folding; I, interaction with other bivalents; D, differential staining. The asterisk denotes the androgenetic origin of YY male 1.

FIGURES

Figure 1. a, Whole synaptonemal complex spread showing 22 bivalents in XX male. b, detail of a bivalent in wild-type male. c, interlocking in wild-type male. d, whole SC spread showing 22 bivalents in wild-type male. e, longest bivalent showing unpaired axial elements in central and subterminal regions, XX male. f, longest bivalent showing unpaired axial elements in subterminal region, YY male. Key: AP, attachment plaques; CR, central region; I, interlock; K, kinetochore; LB, longest bivalent; LE, lateral elements; PL, paired lateral elements; UA, unpaired axial elements. Scale bars represent 3 μm in a, 0.5 μm in b, 3 μm in c-f.

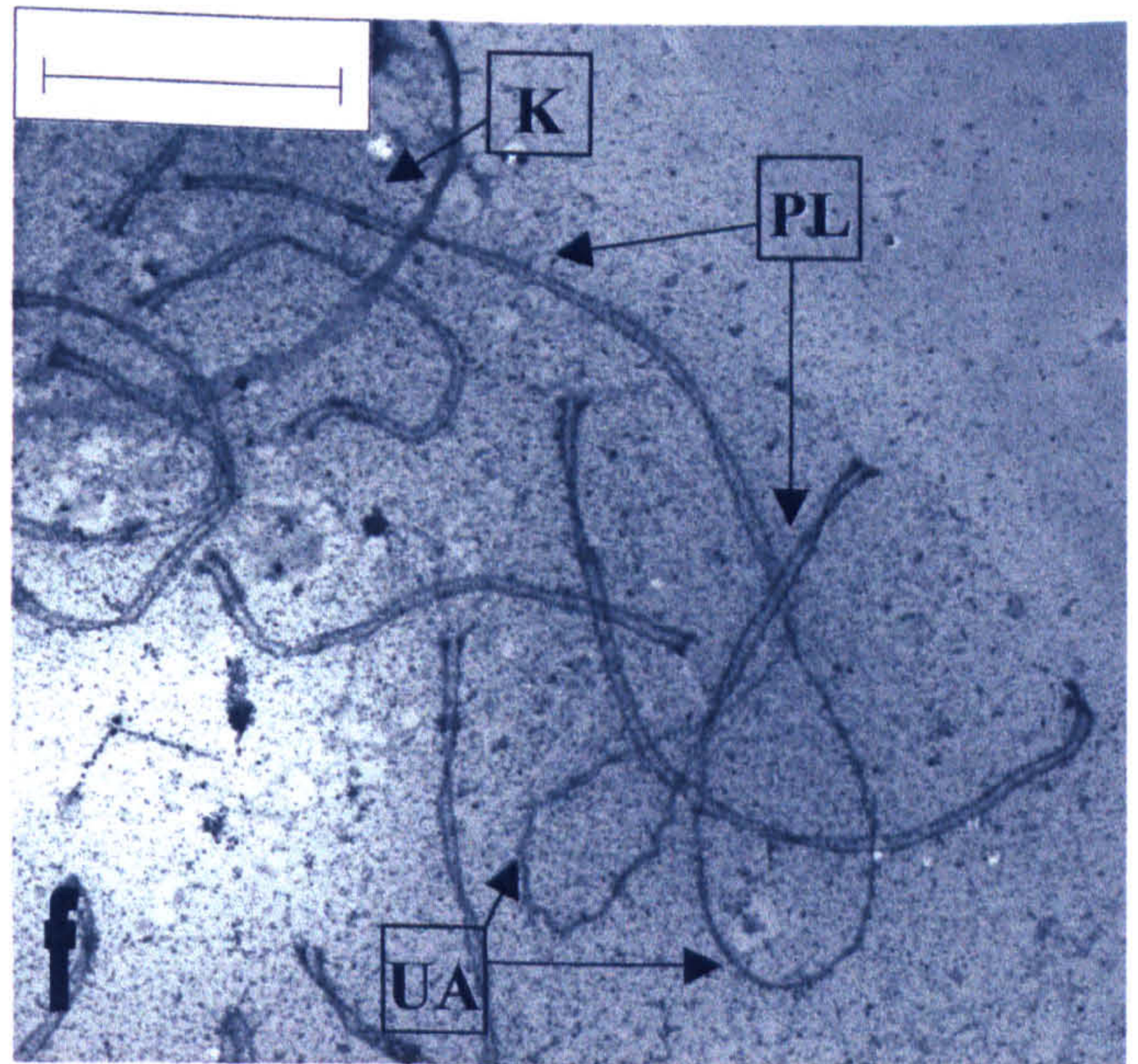
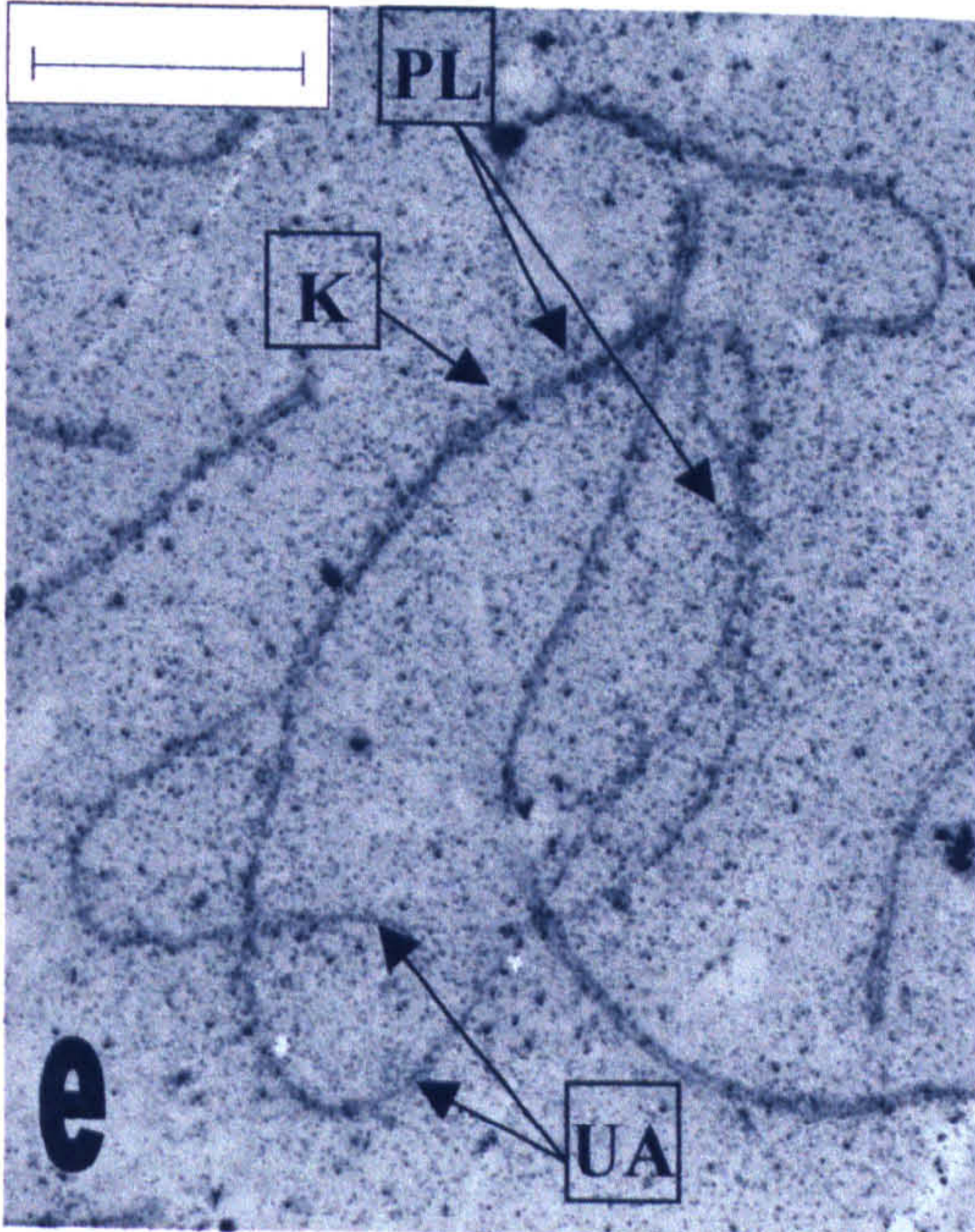
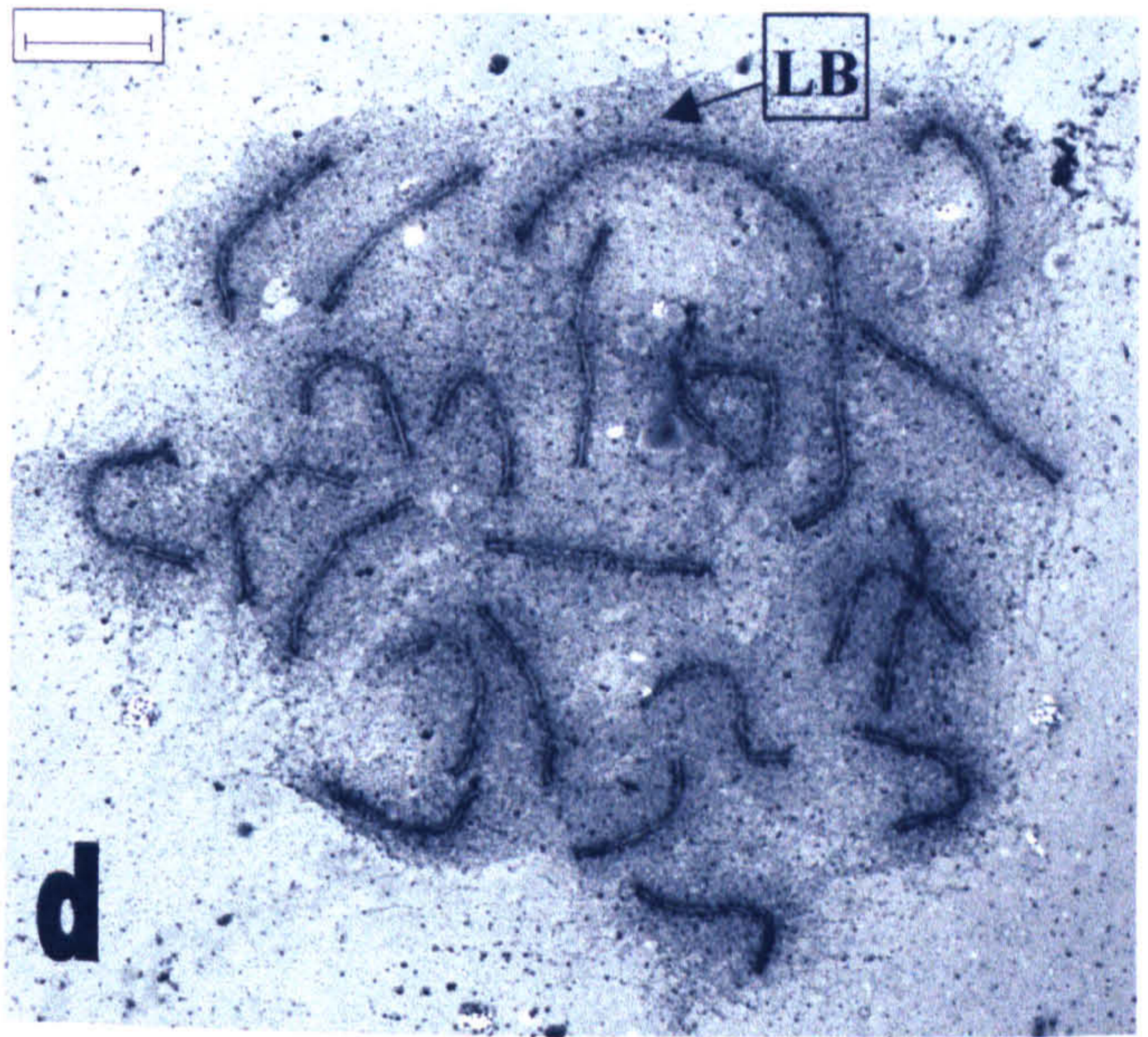
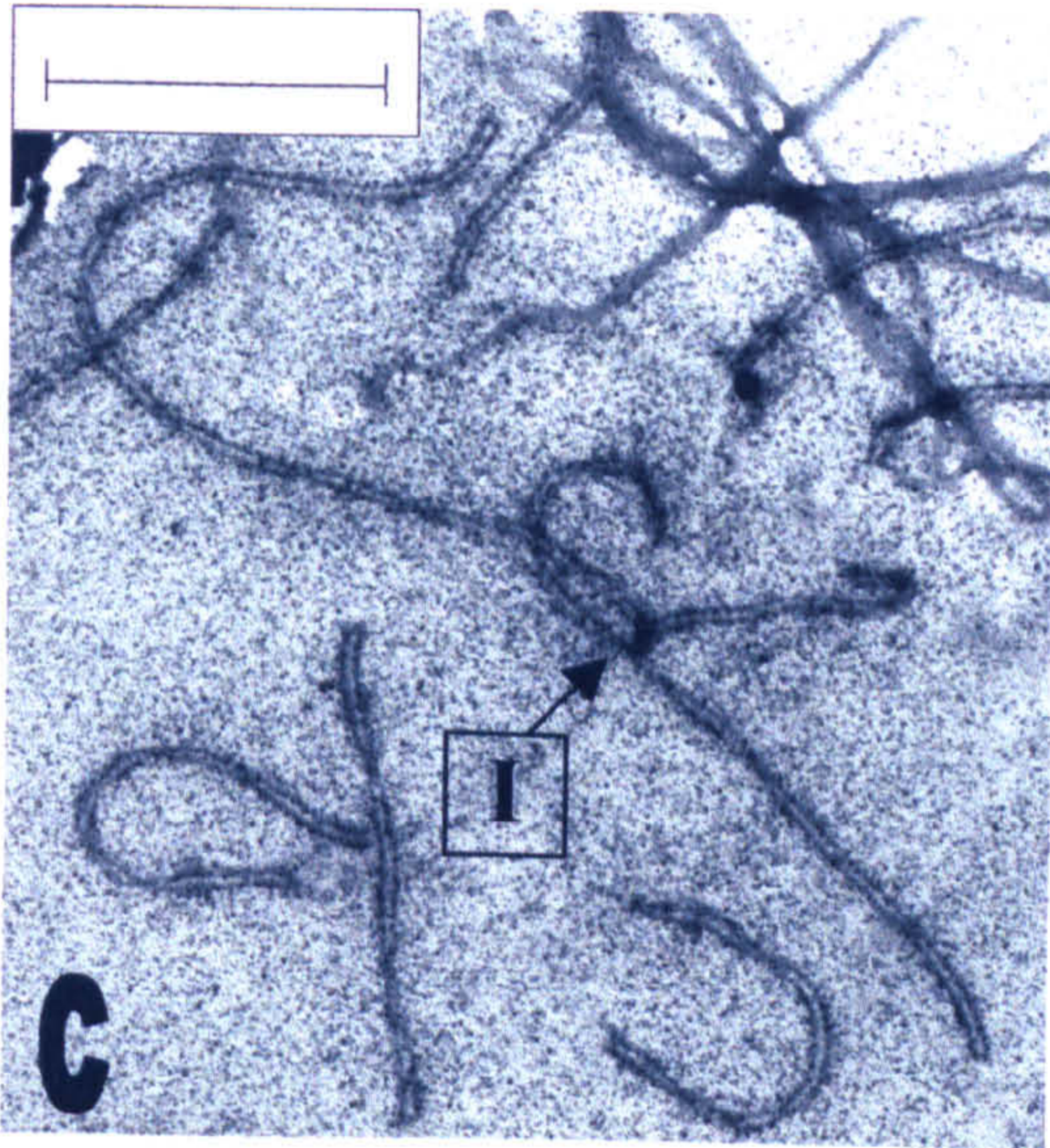
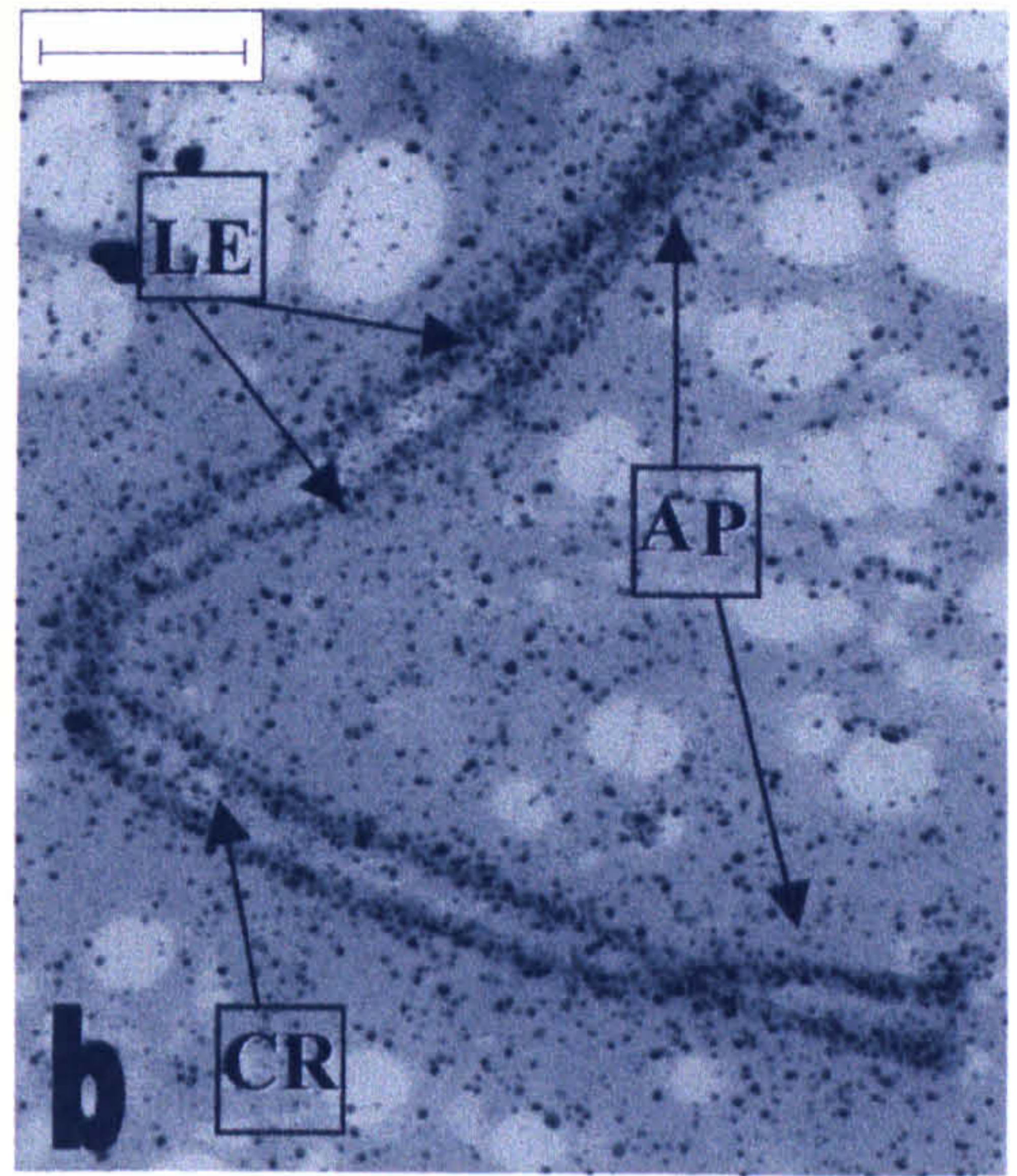
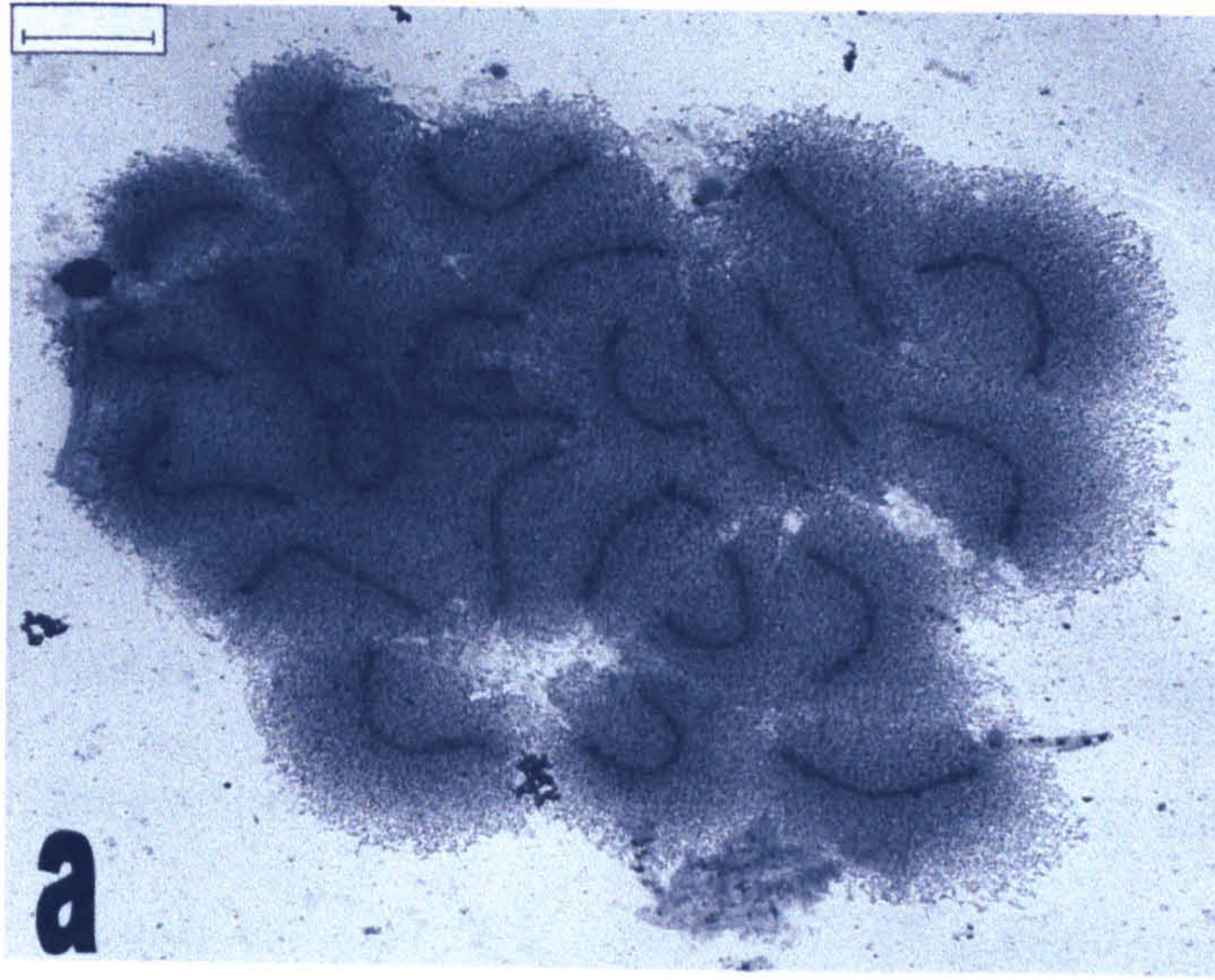


Figure 2. a, Longest bivalent showing unpaired axial elements in the terminal region, wild-type male. b, Longest bivalent showing unpaired terminal region, wild-type male. c, longest bivalent showing unpaired axial elements of different length, wild-type male. d, longest bivalent showing unpaired axial elements of different length in the terminal region, wild-type male. e, fully paired lateral elements in the longest bivalent, YY male. f, self-folding terminal region in the longest bivalent, wild-type male. g, multivalent involving the longest bivalent, wild-type male. h, whole SC spread showing differential staining of the longest bivalent, XX male. Key: F, self-folding lateral elements; K, kinetochore; LB, longest bivalent; PL, paired lateral elements; UA, unpaired axial elements. Scale bar represents 3 μm .

