Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA

BIODIVERSITA' ED EVOLUZIONE
Ciclo XXI

Settore/i scientifico disciplinari di afferenza: BIO-05

TITOLO TESI

Methodological approaches in order to investigate the mechanisms of sexual determination and sexual differentiation in the common toad *Bufo bufo* L. (Amphibia, Anura).

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Esame finale anno 2008

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1.INTRODUCTION

1.1) Sexual determination and sexual differentiation: generalities.

In gonochoristic animals, sexual determination and sexual differentiation are two different but closely related mechanisms involved in the development of the alternative sexual phenotypes. Although often used interchangeably, there is a fundamental difference between sexual determination and sexual differentiation. Sexual determination is referred to mechanisms that direct sexual differentiation, whereas sexual differentiation is referred to the development of the alternative gonadal architectures (testes or ovaries) starting from the bi-potential and undifferentiated gonad.

The different sexes develop because individuals are directed through alternative developmental pathways as a result of the activation of the sex determining genes or as a consequence of an enviromental induction that not only include the sexual differentiation of reproductive organs, but also affects almost every aspect of an organism such as behavior, physiology, and morphology.

All individuals may have the capacity to develop into either sex. This capacity is evident from the ability to experimental sex reverse as in amphibians: depending on the species, genetic males can be experimentally induced to develop ovaries and vice versa with steroid hormonal treatments.

From an evolutionary point of view, sexual determination is considered as a convergent adaptive phenomenon that takes place in accordance with a wide range of alternatives. Starting from an hermaphrodite information the alternative pathways are imposed by the conversion of the analogical signal into the binary signal initiating male or female development.

On the contrary, sexual differentiation is considered as a conservative phenomenon, quite similar in each classes of vertebrate, that leads to the development of testicular or ovarian architectures. For these reasons, is very important to understand the different competences of the somatic and germinal districts and their interactions during the development of the alternative typologies of gonadal architectures.

From the first years of the 1900 the issues about sexual differentiation were analyzed and described resulting in manifold interpretative models that diverge on the attribution of the primary inductive role exerted by the germinal cells or by the somatic cells during male or female gonadal development.

These difference are resumed in few interpretative models. The “symmetric” model argue that the bi-potential condition of the germinal cells is a previous condition before of somatic sexualizing actions: the germinal cells, as passive targets, would be able to differentiate only in presence of a differential somatic inductor derived by different somatic districts .

Another model termed “asymmetric”, recognize at the germinal cells the innate tendency to develop, in absence of somatic inputs, trough a female development. The bi-potent condition of the germinal cells would be the result of a somatic inhibitory signal on a primary sexual development program where female differentiation would be the “default” pathway.

1.2) Sexual determination in the animal kingdom: comparative study in different models organisms.

Sex determination is an integral part of reproduction and an essential process for the evolvement and enrichment of the genome. It has thus been the subject of many studies in reference to species across the entire animal kingdom. From insects to mammals the animals display different strategies to determine the sexual fate: for this reason we can consider the sexual determination as an adaptive mechanism during the evolutionary pathway. Interestingly, data so far accumulated by a variety of model organisms has shown a relative economy in the molecular regulation of sex determination. More specifically, sex determination has so far proven to be the result of one of the following three mechanisms:

1) enviromental action on the embryo at a crucial stage of development. In this kind of mechanism the temperature or better the range of temperatures, determinate the sexual choice. Other enviromental variables such as pH or social variables (the size of an organism relative to other members of its population) can condition the sexual fate.

Concerning enviromental action, the temperature is consider the principal factor of the enviromental sex determination (ESD); in fact this mechanism is also described as temperature-dependent sex determination and the developmental stage of sex determination is referred to as the thermosensitive period (TSP). This kind of mechanism is observed in lower vertebrate classes like fishes and reptiles (in which the body temperature depends by the enviromental temperature);

2) genetic action, when at least one specific gene is considered to be the central regulator, the switch, in a cascade of events leading to the determination of sexual phenotype. We can find this mechanism starting from the analysis of invertebrates and reaching to high vertebrates, but at today there is not sufficient knowledge about these switch genes;

3) the presence of distinct sex chromosomes or gonsome. The identical pair may be present in both males (birds) and females (mammals) and their major sex determining gene may be either known (the mammalian SRY) or still suspected.

Although sex determination has been suggested to promote specific function at a universal level, such as selective cell proliferation (Mittwoch) or steroid hormone accumulation (Howard), this issue remains debatable. Interesting, the animal kingdom displays of relative limited regulatory patterns to reach the determination of the sex; this fact could be the result of a single general regulatory scheme, at least in vertebrate, potentially involving or incorporating both hormonal elements and dosage compensation epigenetic regulatory phenomena.

The description and the study of different models organisms is a primary necessity to value the general regulatory scheme and to reach high levels of knowledge among the entire animal kingdom. We must also consider that all the information about the ancestral classes of vertebrate like fishes, amphibians and reptiles could give us the keys to understand the complexity of this mechanism in mammals and maybe to understand some dysfunctions in the human being connected with an abnormal development of the gonads.

1.3) Invertebrate

A variety of sex determining mechanisms are known for insect including male and female heterogamety, haplodiploidy, parental genome loss and systems with X chromosome elimination (Beukeboom,1995; Beukeboom et al.2000)[1,2].
The comparison between invertebrate and vertebrate model organisms share some interesting analogy to define the sexual choice and in the sex determining pathways.

a) Hymenoptera

One of the most interesting model to consider is certainly the class of hymenoptera. This class count about 200'000 spices spread in all the world and all of these are showing the mechanism of haplodiploidy.
The dominant and ancestral mode of sex determination in the hymenoptera is arrehenotokous parthenogenesis: a subset of haplodiploidy in which diploid females develop from fertilized eggs and haploid males develop from unfertilized eggs (George et al.2008) [12].

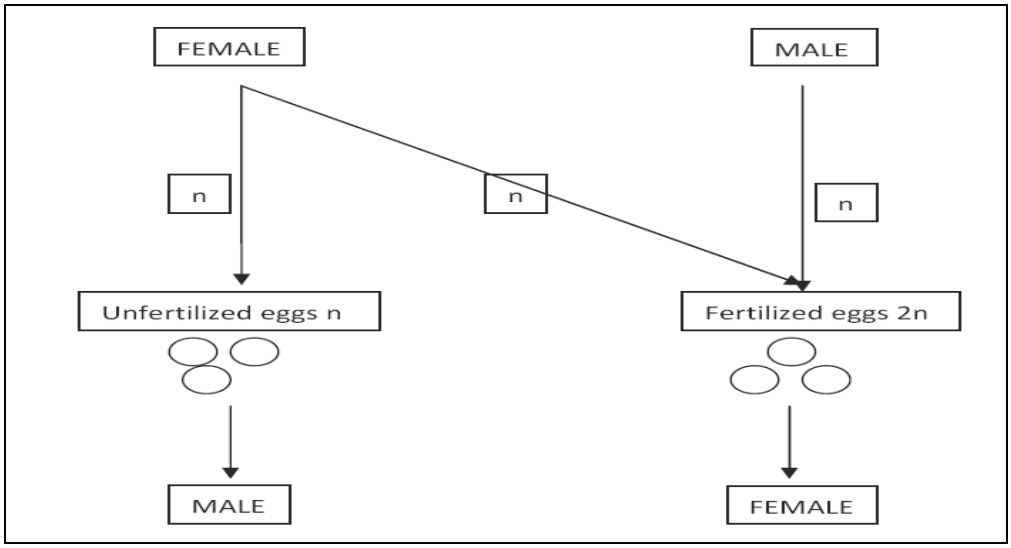

Figure 1.1: haplodiploidy reproduction. In Hymenoptera, unfertilized eggs develop into uniparental haploid males whereas fertilized eggs into biparental diploid females.

This mode of reproduction holds a specific position in sex determination because uniparental males inherit a random half of the maternal genome, while females inherit both the maternal and paternal genes rendering any chromosomal-based sex-determining system impossible (Bull, 1983) [3]. The best-studied genetic example of sex determination in haplodiploids can be attributed in some hymenopteran insects (bees, wasps, ants) to a single, highly polymorphic sex locus in which a single locus with several alleles directs sexual development (Cook, 1993)[4]: the so-called single locus complementary sex-determining mechanism (*sl*-CSD).
For the honeybee, *Apis mellifera*, the estimated numbers of sex-determining alleles segregating in populations range from 11 to 19.

Under single-locus complementary sex determination (*sl*-CSD), eggs that are heterozygous at the sex locus develop as females while hemizygous and homozygous eggs develop as haploid and diploid males, respectively. Diploid males may be unviable, sterile, or functionally reproductive (Stouthamer *et al*, 1992; Cowan and Stahlhut, 2004) [5,6], and they are produced under inbreeding, or in populations with low sex allele diversity. While sterile or unviable diploid males constitute a genetic load associated with *sl*-CSD (Werren, 1993; Cook and Crozier, 1995) [7,8], the recent finding of functionally reproductive diploid males in the vespid *Euodynerus foraminatus* suggests that the production of diploid males need not always represent a reproductive dead-end (Cowan and Stahlhut, 2004) [6].

The population dynamics of sex-determining alleles have some notable parallels to the self-incompatibility loci in plants and fungi. When an allele is shared between pollen and pistil or between fungi with the same mating type then an incompatibility response will follow. Typically, these systems are controlled by a single genetic locus having multiple allelic versions or specificities. Investigations into the molecular bases have revealed diversity in the mode of recognition and rejection in these systems.

The 1453 base long *csd* sequence consists of nine exons and contains an open reading frame (ORF) of 385 amino acid residues. Sequence comparisons indicated that CSD is a member of an arginine-serine-rich (RS) protein. CSD protein is a member of RS protein; previous studies have suggested that proteins with an arginine-serine (RS) rich domain are involved in protein-protein interactions and have a dominant role in constitutive and regulated pre-mRNA splicing and metabolism.

Proteins with RS domains fall into two distinct groups: those with an RNA recognition motif (RRM) at its N terminus that binds directly to RNA, the SR proteins (Hastings and Krainer, 2001) [9], and those that are structurally distinct from the SR proteins, the so-called SR-related proteins. The *csd* gene constitutes a divergent member of the SR-related protein family that does not contain any RNA binding domain.

CSD protein shows some sequence identity to various RS domains that include some conserved SR protein members involved in pre-mRNA splicing, mRNA processing and RNA binding; highest similarity was found for its C terminus to *Tra* (*transformer*) protein which achieves female-specific splicing of *dsx* transcripts in the sex-determining cascade of *Drosophila melanogaster*. No homology, however, is found for the N terminus that is supposed to be conserved among RS protein orthologs, supporting its function in the specialized process of complementary sex determination.

SR-related proteins can generate multimeric proteins that govern development by specialized splicing. A challenging problem is to determine how the polymorphic signal of various allelic combinations of *csd* is transformed into the binary switch initiating male and female development. Results with RNAi show that a heterozygous allelic composition encodes a functional product that initiates female development; repression of the *csd* transcript in female honey bees led to male development, providing conclusive evidence that *csd* controls sex determination.

The hemi or homozygous allelic composition produces a nonfunctional product that regulates male development by default.

The model proposed is that only the combination of polypetides from different alleles yields an active heteromeric protein complex. Most putative amino acid differences that characterize the

various alleles are found in the very variable C terminus, in the RS domain, in the proline-rich region, and in a hypervariable region between them. RS domains and proline-rich regions in general have protein binding abilities suggesting that differences in the amino acid sequence may result in modification of protein-protein interactions that are critical in the regulation of splicing.
CSD does not constitute a universal explanation in the hymenoptera, however, as it is absent from a number of species.
Homozygotes at *csd* have zero or near zero fitness in honey bees and most other hymenopterans with CSD, and the sex locus is therefore excepted to be under both balancing selection (balancing selection refers to forms of natural selection which work to maintain genetic polymorphisms ,or multiple alleles, within a population) and diversifying selection (disruptive selection, also called diversifying selection, is a term used to describe changes in population genetics that simultaneously favor individuals at both extremes of the distribution. When disruptive selection operates, individuals at the extremes contribute more offspring than those in the center, producing two peaks in the distribution of a particular trait). This means that selection should maintain diverse allelic variants within a population, with rare alleles enjoying a selective advantage over common alleles. This balancing selection is reflected in honey bees by a surfeit of nonsynonimous nucleotide changes across the different *csd* alleles, particularly in young alleles. Nonsynonymous substitution are by definition necessary in the formation of novel functional sex alleles, and the finding of an excess of these classes of substitution in the RS domain is consistent with both the specification of functional allele differences in this area and a history of selection for novel alleles. The *csd* locus supports low levels of recombination, a feature that can lead to the long-term preservation of difference among alleles. In contrast, genomic areas flanking the sex-determining locus have abnormally high recombination rates, which may reduce potential conflicts between *csd* and neighboring genes (Hasselmann et al.2006) [10]. The discovery of the *csd* gene will likely lead to elucidation of the molecular mechanism by which *csd* controls sex determination. *csd* shows moderate homology to *transformer*, one of the major sex-determing gene in *Drosophila melanogaster* that also contains an RS domain. In *Drosophila*, *transformer* is involved in female-specific splicing of the *doublesex* transcript, which initiates the female development pathway. The conservation of *transformer* function between *Drosophila* and *Apis*, if confirmed, would support the hypothesis that most evolution in sex-determining pathways has occurred at or near the beginning of the pathway, with downstream components more conserved in function (George et al.2008) [11].

b) Dipterans (Drosophila melanogaster)

Certainly, the most investigated model organism in genetics and genomics is the fruit-fly *Drosophila melanogaster*. As reviewed by Gilbert,2000 [17] *Drosophila* sex determination is due by the presence of a single feminizing switch gene called sex-lethal (*sxl*) in response to the balance of female determinants on the X chromosomes versus male determinants on the autosomes (X:A ratio). Normally, fruit-flies have either one or two X chromosomes and two sets of autosomes: the presence of a single X chromosome in a diploid cell (1X:2A) develop a male phenotype while two X

chromosomes in a diploid cell (2X:2A) lead to a female phenotype (Bridges 1921, 1925) [14,15]. Thus, XO *Drosophila* are sterile males.

In XY cells, *sxl* remains inactive during the early stages of development (Cline 1983; Salz et al. 1987) [16,19]. In XX *Drosophila, sxl* is activated during the first 2 hours after fertilization, and this gene transcribes a particular embryonic type of *sxl* mRNA that is found for only about 2 hours more (Salz et al. 1989) [20]. Once activated, the *sxl* gene remains active because its protein product is able to bind to and activate its own promoter (Sánchez and Nöthiger 1983) [21]: *sxl* regulates the splicing of its own pre-mRNA with a typical positive feedback loop.

This female-specific activation of *sxl* is thought to be stimulated by "numerator proteins" encoded by the X chromosome. These constitute the X part of the X:A ratio. Cline (1988) [18] has demonstrated that these numerator proteins include Sisterless-a,b,c. These proteins bind to the "early" promoter of the *sxl* gene to promote its transcription shortly after fertilization.

The "denominator proteins" are autosomally encoded proteins such as Deadpan and Extramacrochaetae. These proteins block the binding or activity of the numerator proteins (Younger-Shepherd et al. 1992)[22]. The denominator proteins may actually be able to form inactive heterodimers with the numerator proteins. It appears, then, that the X:A ratio is measured by the balance between X-encoded activators genes at the numerator and autosomally encoded repressors genes of the promoter of the *sxl* gene in conjunction with maternally derived products. All this take place early in development, leading to the activation of the *sxl* gene through an early promoter only in females.

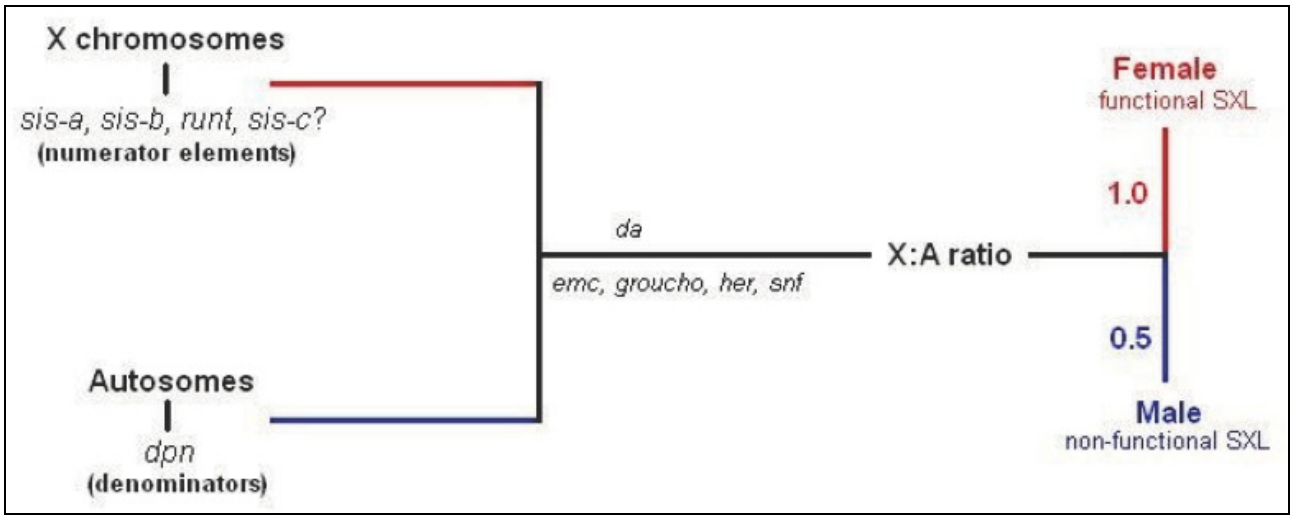

Figure 1.2: the X:A ratio determines sex in *D.melanogaster.* In *Drosophila melonogaster* sex is the result of the X:A ratio as result of the balance between the X numerator elements and the autosomal denominators in the presence of several maternally derived proteins. An X:A ratio of 0,5 leads to nonfunctional SXL and male development, whereas an X:A ratio of 1 maintains SXL in its active state (early promoter) to female development (from Manolakou, 2006)[47].

This early form of the SXL protein absent in males, orchestrate a different splicing activating a "late" promoter on the *Sex-lethal* gene that is now transcribed in both males and females. Only in females, trough an autoregulatory feedback loop, that *sxl* manages to keep itself in an active and functional state through this alternative sex-specific splicing. The autoregulatory loop exemplifies

how an early developmental decision can be “remembered” for the rest of development, even after the initial signals that established the decision have long disappeared.

Analysis of the cDNA from *sxl* mRNA shows sex specific difference: the *sxl* mRNA of males differs from *sxl* mRNA of females (Bopp et al. 1991) [23]. While the male specific *sxl* mRNA transcript is nonfunctional, the female-specific *sxl* message encodes a protein of 354 amino acids. The major difference between the alternative transcript is that male-specific *sxl* transcript contains an early translation termination codon (UGA) as consequence of the alternative splicing of *sxl* mRNA: splicing control by *sxl* allows the production of a functional protein product only in presence of early promoter. Protein–protein interactions, such as competition between normal and inhibitory subunits for dimer formation, can be triggers for controlling developmental switches. The protein made by the female-specific *sxl* transcript contains two regions that are important for binding to RNA. These regions are similar to regions found in nuclear RNA-binding proteins. Research have shown that there are two targets for the female-specific SXL protein (Kelley et al.1997) [24]. One of these targets is the pre-mRNA of *sxl* itself. The second is the pre-mRNA of the downstream target gene on the pathway, *transformer.*

The *sxl* gene regulates somatic sex determination by controlling the processing of the *transformer* (*tra*) gene transcript. The *tra* message is alternatively spliced to create a female-specific mRNA as well as a nonspecific mRNA that is found in both females and males. Like the male *sxl* message, the nonspecific *tra* mRNA contains a termination codon early in the message, making the protein nonfunctional (Belote et al. 1989) [25]. The female-specific *tra* product acts in concert with the product of the *transformer-2* (*tra2*) gene to help generate the female phenotype. *Tra* forms a heterodimer with the *Transformer-2* (*Tra-2*) protein that modulates the splicing of other two genes: double sex (*dsx*) and fruitless (*fru*). Once that SXL active state has been established, it then goes on to regulate a series of other proteins that control female development as *tra/tra2*, leading finally to the two alternative products of the *doublesex* gene (*dsx*), *DSXf* and *DSXm*.

The doublesex (*dsx*) gene is active in both males and females, but its primary transcript is processed in a sex-specific manner (Baker et al. 1987) [13]. This alternative RNA processing appears to be the result of the action of the *transformer* gene products on the *dsx* gene. If the *Tra-2* and female-specific *Tra* proteins are both present, the *dsx* transcript is processed in a female-specific manner. The female splicing pattern produces a female-specific protein that activates female-specific genes (such as those of the yolk proteins) and inhibits male development while if functional *Tra* is not produced, a male-specific transcript of *dsx* is made. This transcript encodes an active protein that inhibits female traits and promotes male traits. If the *dsx* gene is absent, both the male and the female primordia develop, and intersexual genitalia are produced.

This chain of alternative genes splicing drives the development of the appropriate sex. It is interesting to highlight how the Y chromosome, present in males, is not involved in determining sex, it only helps the correct completion in differentiation of the male germline during the spermatogenesis. According to this model (Baker 1989) [11], the result of the sex determination cascade comes down to what type of mRNA is going to be processed from the *dsx* transcript. If the X:A ratio is 1, then *Sxl* makes a female-specific splicing factor that causes the *tra* gene transcript to be spliced in a female-specific manner. This female-specific protein interacts with the Tra2 splicing factor to cause the *doublesex* pre-mRNA to be spliced in a female-specific manner. If the *doublesex*

transcript is not acted on in this way, it will be processed in a "default" manner to make the male-specific message.

c) Nematodes (Caenorabditis elegans)

The current model of sex determination in *C.elegans* begin with a start signal that is based on the number of X chromosomes in a diploid animal. In 1949, Nigon demonstrated that *C. elegans* used an XX/XO sex chromosome system, and the importance of the rapport between X chromosomes and autosome was clarified by Madl & Herman (1979) [26].

The wild-type sexes in diploid *C.elegans* are the self-fertile hermaphrodite, which has two X chromosomes and the male which has one X chromosome (X0).Therefore males have an X:A ratio of 0.5 and hermaphrodites a ratio of 1.0.

In order to equalize the amount of gene products between the sexes, the hermaphrodite transcript level of the X-linked genes are reduced by half (Meyer and Casson, 1986; Donahue et al.,1987) [27,28]. This differ from how dosage compensation is achieved in *Drosophila* where the single X chromosome in the male is twice as active as each of the two X chromosomes in female.

C.elegans sex determining system provides another opportunity for examining the regulatory interaction between a small set of genes that control the choice of the sexuality.

The development depends by the worm's ability to interpret the balancing between determinants on the autosomic chromosomes and determinants on the sexual chromosomes: the X:A ratio regulates the activity of *xol-1* via the combined action of a set of "numerator" or X-signal elements (*fox-1, sex-1*) on the X chromosome and "denominator" (*sea-1,sea-2*) autosomic signal elements elsewhere in the genome.

A potential numerator element is called *fox-1* (feminizing locus on X) and another X-linked numerator element is *sex-1*(signal element on X). *sex-1* and *fox-1* have additive effects in establishing the X:A ratio. SEX-1 (Signal Element on X) and the post-transcriptional protein FOX-1 (Feminizing locus On X), in association with others elements that are still not identified, inhibit the expression of X0L-1 (X0 lethal) the protein that is present in male pathway.

Concerning the denominators elements, *sea* genes denominators have yet been identified. Thus, somatic sex, germ-line sex and X-chromosome gene expression are controlled by the dosage of several genes on the X chromosome which seem to act additively by a mechanism that is still not clear. All numerator elements act to control the expression of a single master switch gene, *xol-1* (X0 lethal) (Nicoll et al,1997) [29].

According to the model, *xol-1* expression promote male development and allow for male X-transcript levels in X0 males while it is inactive in XX animals to allow for the hermaphrodite fate as well as a lowering of X-linked transcript levels. In male animals with genetic asset X0, *xol-1* appears to act by repressing the activity of the three *sdc* (sex and dosage compensation) genes. *xol-1* seem to be regulated in a number of ways: one of these is the regulation by SEX-1. SEX-1 acts as a transcriptional repressor of *xol-1* by directly binding to its promoter (Carmi et al.,1998) [30]. All these genetic elements play a role in both sex determination and dosage compensation. In contrast, all factors acting epistatically downstream of the *sdc* genes are involved in either dosage compensation or sex determination, but not both.

In somatic sex determination the *sdc* genes promote the hermaphrodite fate in XX organisms, at least in part, by lowering the transcript level of the male specific gene, *her-1* (*hermaphrodization*) (Schauer and Wood,1990) [31]. *her-1* is the first gene in the pathway that responds most directly to the primary sex determining signal (*xol-1*) that depends by the ratio of X chromosomes. The other genes respond indirectly, so that we can define one set of genes in XX animals and another one established in X0 animals. The current model of protein interactions involved in somatic sex determination differ in X0 and XX organisms by the specific bounding of the protein HER-1 at the cell membrane protein TRA-2 only in males.

The binging inactivates TRA-2 allowing the FEM proteins to exert their inhibitory influence on TRA-1. With TRA-1 repressed, the cell is able to take on the male fate. In organism with XX chromosomes asset, HER-1 is not present and TRA-1 is able to bind DNA promoting the female development. *tra-1* is thought to be the terminal regulator of somatic sex determination by functioning as a transcription factor that regulates sex specific genes. *tra-1* locus makes two mRNA that encode two different proteins (Zarkower and Hodgin, 1992) [32]. The smaller protein contains two zinc finger motifs while the larger contains five, and only the larger is capable of binding DNA in vitro (Zarkower and Hodgin, 1993) [33] supporting the idea that TRA-1 functions as a transcriptional regulator that controls the expression of sex specific genes. *Mab-3* (*male abnormal*) function downstream of tra-1.

Another mechanism is responsible for the specification of the sex in the germinal cells.

Since the *C.elegans* XX animal makes sperm and oocyte in each of its ovotestes, it is reasonable to assume that additional levels of genetic control are needed in germline compared to the soma. One of the major obstacles that hermaphrodite must addressed is how a differentiated male tissue can be made in a female soma while the environment remains competent for subsequent oocyte production. Spermatogenesis occurs for a short period of time, before switching to oogenesis, implying that the regulation of genetic factors involved in promoting sperm and oocyte production must change at this point. Gamete production in the XX germ line is divided into two temporal phases: spermatogenesis and oogenesis. The principal issue is about how the male fate is reached in organisms with a balancing of chromosomal determinants culminating in a female X/A ratio. First of all, the terminal regulator in the germ line differs from that in somatic districts. *tra-1* is the terminal regulator only in somatic cells and function by promoting the female fate when active and allowing for a male soma development when absent. In the germ line, epistatic analysis suggest that *tra-1* is not the terminal regulator although is necessary for continued spermatogenesia.

Two others genes also work with the *fems* to promote spermatogenesis namely *fog-1* and *fog-3*. *fem* and *fog* genes termed *tgfs* must be active to promote spermatogenesis and their activity is dependent upon the inactivity of *tra-2*. Active *tra-2* serves to repress at least one of the *tgfs*. A possible mechanism for *tra-2* inactivation in the germ line involve in hermaphrodite another fog gene: *fog-2*.

Another mechanism regulating *tra-2* in the hermaphrodite may involve a signal emanating from the somatic cells. These cells may secrete an inhibitory ligand for TRA-2 similar to the action of HER-1 in the male soma. The transient down regulation of *tra-2* thereby allows the *tgfs* to promote a brief period of spermatogenesis sufficient for approximately 150 sperm to be made per

gonad arm, after which a switch is made to oocyte production. This required that one of more *tgfs* genes be turned off and oocyte promoting factors turned on. At least, in part this may be accomplished by repressing translation of *fem-3* through its 3' UTR. Six other genes (*mog1..6*) are also involved in the switch from sperm to oocyte production (Graham et al.,1993) [34]. Spermatogenesis in the hermaphrodite could be the result from a transient repression of *tra-2* activity in the XX larval germline, which permits sufficient *fem* gene activity to promote a phase of spermatogenesis (Hansen et al.1999) [35].

1.4) Vertebrates

According to experimental evidence, vertebrates display different strategies leading to the development of alternative sexual phenotype. Among vertebrates, we can find classes in which coexist different mechanisms of GSD (such as XX/XY,ZZ/ZW) and other in which coexist GSD, TSD and also hermaphrodites. From an evolutionary point of view we can argue that at lover evolutionistic levels hermaphrodites and enviromental sex determination are represented and then, at higher levels, they are replaced by genetic sex determination.

a) Fishes.

There are numerous species of fishes in the animal kingdom, with estimation as to their current number reaching a mean price of 25000. Among such a variety of living organisms, research has been focused on relatively few specific model organisms each of which has been considered representative of the reproductive physiology of several other closely related species. Among the mechanisms observed one may refer to:
1- the presence of true hermaphrodites: a strategy usually associated with lower evolutionary levels,
2-temperature dependent sex determination,
3-genetic sex determination and sex chromosomal sex determination (XX/XY or ZZ/ZW patterns).
It will be possible that this different kinds of mechanism have evolved separately through the evolution.
While hermaphrodites is not uncommon in worms and insects, it is rarely seen in vertebrates. In birds and mammals, hermaphroditism is usually a pathological condition causing infertility. The most common vertebrate hermaphrodites are fishes, which display several typology of hermaphroditism (Yamamoto, 1969) [36].
Some fishes, however, are gonochoristic; that is, they have a chromosomally determined sex that is either male or female or an enviromental sex determination.
Hermaphroditic fishes species can be divided into three groups. The first are the synchronous hermaphrodites, in which ovaries and testicular tissues exist at the same time and in which both sperm and eggs are produced. One such species is *Servanus scriba.* In nature and in aquaria, these fish form spawning pairs. As soon as one of the fish spawns its eggs, the other fish fertilizes them. Then the fish reverse their roles, and the fish that was formerly male spawns its eggs so that they can be fertilized by the sperm of its partner. In other hermaphroditic species, an individual

undergoes a genetically programmed sex change during its development. In these cases, the gonads are dimorphic, having both male and female areas. One or the other is predominant during a certain phase of life.

In protogynous "female-first" hermaphrodites, an animal begins its life as a female, but later becomes male. The reverse is the case in protandrous "male-first" species. In protogynous fishes all the organisms born as females and then, in adult age, invert the sex into males. One of the most explicit examples is the case of *Labroides dimidiatus*. In this specie exist an α-male: it dominates a little number of adult females and youth organisms that live in a restricted area. Also the adult females present a precise hierarchy: at the mating the male fruitful the dominant female, then the second one, then the third one following the hierarchy supremacy. If the dominant males die, the dominant female turn into male with a mechanism of complete sexual inversion that take place during only one week. After this period the ex dominant female begin its new life as dominant male.

An evolutionistic hypothesis to explain the protogynous model is that it would be favored by the natural selection when the females have a reproductive advantage connected with the short size and when the male has big size as in labridi. The explanation of this statement is referred to the model of selective advantage in size. Males with big size are advantaged in mating and so at the little ones do not invert the sex remaining females. Only after a long period of development and a great increment in size they will be able to compete against the dominant male. So, we can say that in this case of hermaphroditism, sexual dimorphism is represented by females with short size and male with big size.

We can find also other species that display a proterandric hermaphroditism (when the females are bigger than males). This fact could find an evolutionistic answer considering the correlation between number of eggs and size. The size of the eggs is steady in each species; if a female display a bigger size it is able to product more eggs and so is able to increase its fitness.

Concerning species which display temperature sex determination TSD, there are no consistent genetic differences between sexes. The earliest ontogenetic difference between sexes is an environmental one because the ambient temperature during sensitive period of early development irreversibly determines phenotypic sex and, therefore, the sex ratio.

In fishes, the first evidence of TSD was obtained investigating the Atlantic silverside, *Menidia menidia*. Fishes with TSD have readily been grouped according to three patterns of sex ratio response to environmental temperature during the thermosensitive period: more males at high temperature; more males at low temperature; and more males at extreme (high and low) temperatures. In any case, the presence of TSD in a given species is not incompatible with the existence of a different genotype. However, too often assignment of TSD in many fish species has proceeded regardless of evidence such as the presence of sex chromosomes, which is strongly indicative of GSD. Thus, evidence to support the presence of TSD has been obtained in many cases using temperatures in the laboratory that the species will rarely experience in nature (Conover, 2004) [37].

It has been pointed out that observed sex ratio shifts under these circumstances might be the consequence of thermal effects on GSD (GSD+TE) rather than proof of the presence of TSD. Thus, there is concern regarding the actual prevalence of TSD in fish. In particular, to discern true cases

of TSD from GSD+TE (Conover, 2004) [37]. Nevertheless, the existence of TSD in fishes is now widely accepted, assumed to be widespread and expected to be found in more species as new studies become available.

Finally, GSD in teleost fishes display an amazing diversity of sex-determination systems. Male heterogamety and female heterogamety are sometimes observed within the same genus and even the same species. More complicated systems can involve multiple sex chromosomes and multiple gene loci (influence from autosomal loci on sex determination and polyfactorial sex determination).

For example, in platyfish (*Xiphophorus maculatus*), the genome display three different kinds of sexual chromosomes: X, Y e W. This permits a great number of sexual chromosomes pairs in contrast to the classical vision that is based on the segregation of a single sex chromosome pair that give an homogametic and an heterogametic sex. The WX, WY, XX pairs give a female phenotype whereas XY and YY pairs develop a male phenotype (Schartl et al.2004) [38].

No sex determining genes are detected; research is focused on genes that are located on the W chromosome because all the organisms that carry a W are female.

Another model organism, which display a chromosomal sex determination is the teleost medaka fish, *Oryzias latipes*.

Sex determination in this organisms is founded on male heterogamety as in the mammals but, in contrast to the situation observed in humans, the medaka Y chromosome is very similar to the X; there is no cytogenetic difference between X and Y and X-Y pairing occurs along almost the complete chromosome length.

This suggests that the male-determining region on the Y chromosome should be relatively small. A putative sex determining gene in medaka is *DMY*: it is expressed exclusively in XY males because it is linked at the Y chromosome. The *DMY* gene contain a DNA-binding domain called DM domain which is also present in some proteins involved in sex determination in *Drosophila melanogaster* (DSX) and *C. elegans* (*MAB3*).

DMY is homolog to *DMRT1*, a transcription factor involved in male development in other vertebrates (humans): it should represent the result of a duplication of *DMRT1*. The sequence of *DMY* highlight a higher substitution rate if compared to the autosomical maternal gene *DMRT1*. This evidence support the Y erosion theory and it is reasonable to think that *DMY* and *DMRT1* act as necessary determinants in the male developmental pathway. *DMY* is shown to be essential for male medaka development, as demonstrated by spontaneous XY female (sex reversal) that produced a truncated DMY protein as a result of a single insertion in exon 3. A second typology of XY female presented a very low level of expression of *DMY*. *DMY* expression was detected only in the somatic cells surrounding the germ cells in XY embryos. Taken together, the results of Matsuda *et al.* 2002 [39] indicate that *DMY* is a Y-specific gene required for male development in the medaka fish. In female specimen, the role of the aromatase is central although its induction is not under temperature control but under genetic control. The specific expression of gene like FIGa (factor in germ line a) only in females could be related with the genetic induction of the aromatase.

b) Reptiles.

Two prevailing paradigms explain the diversity of sex determining models in reptiles. Many researcher, consider genetic and enviromental sex determining mechanism to be fundamentally different and that one can be demonstrated experimentally to the exclusion of the other. The conventional view is that these mechanisms are mutually exclusive and they can therefore be viewed as discrete variables. Other researcher argue that no clear boundaries exist between them and that is probable that all sex determining mechanisms have some genetic component.
Recent research on the genes involved in sex differentiation in alligators and turtles with TSD which demonstrates remarkable homology in structure, function and expression of the sex differentiation genes of mammals and reptiles, lends considerable support to that view. Genetic and enviromental sex determination in reptiles should be seen as a continuum of states represented by species whose sex is determined primary by genotype, species where genetic and enviromental mechanisms coexist and interact in lesser or greater measure to bring about sex phenotypes, and species where sex is determinate primarily by environment. In particular exist an intra-generic distribution within some families of enviromental and genetics modes of sex determination and the apparent interaction of both models within some species. In contrast with both mammals and birds, reptiles shows an impressive array of sex determining modes comparable to the variety observed in fishes and frogs. Male heterogamety (XY or XXY) is known in turtles, female heterogamety (ZW, ZZW or ZWW) is known in snakes and both are known in lizards (Graves,2001) [40]. Many species display GSD in the absence of any heteromorphy in the sex chromosomes. Many others have temperature dependent sex determination TSD a form of enviromental sex determination.
In TSD sex determination, the temperature induce the sexual choice during a particular period known as thermosensitive period (TSP). It is during this period that a very specific enzyme enters into the equation. Aromatase, a cytocrome P450 enzyme, is responsible for the conversion of androgens into estrogens and it is common among many organisms: it acts as an important factor in sexual development.

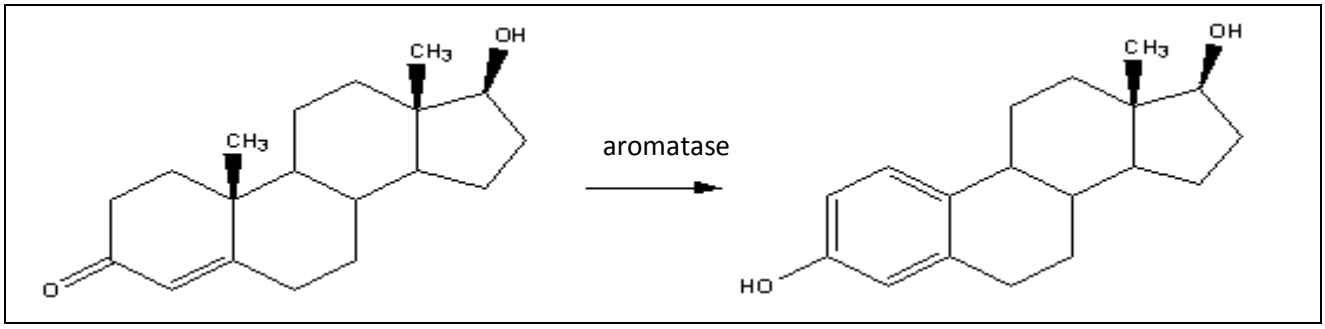

Figure 1.3: aromatase. Aromatase is an enzyme of the cytochrome P450 superfamily that allows the conversion of androgens into estrogens.

In reptiles while steroidogenesis begins very early, prior even to the thermosensitive period, aromatase activity remains universally low. With the onset of the thermosensitive period aromatase activity seems to increase in certain temperatures, which vary for each species. In

marine turtles higher temperatures cause an increment of aromatase activity, whereas in lower temperatures aromatase remains low. The two different levels of aromatase drive the development of the undifferentiated gonad through the female or the male pathways. Once the thermosensitive period is over and the fate of the gonad has been established, further changes in temperature seem to have no effects.

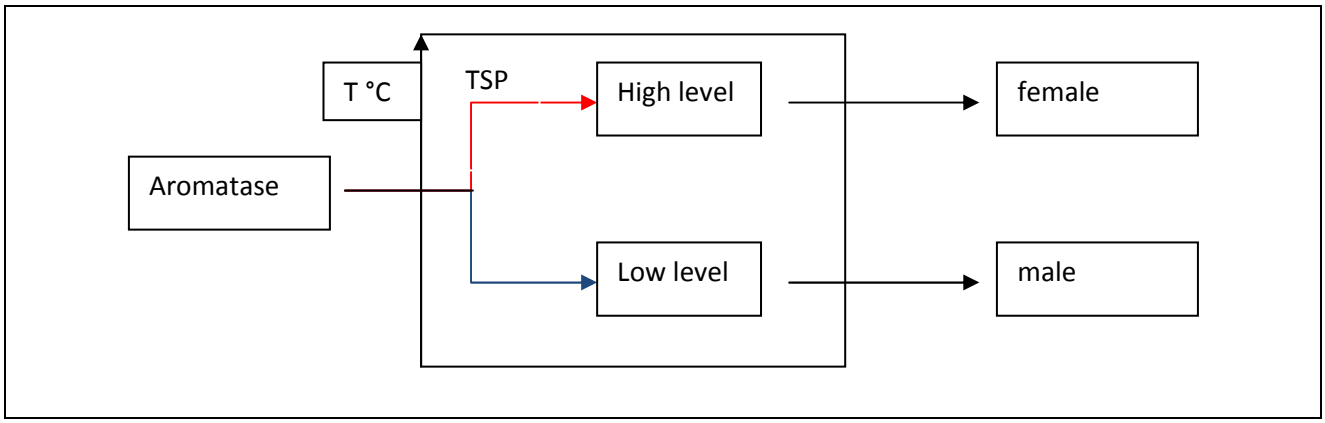

Figure 1.3bis: aromatase and sexual determination. During TSP aromatase levels are regulated by the temperature. Higher temperatures increase the aromatase activity developing ovarian whereas lower temperatures decrease the aromatase activity developing testes.

So, in many species we can consider GSD and TSD as transitional forms. An example provide from the study of the interaction between GSD and TSD in *Pogona vitticeps*. This lizard shows a ZZ/ZW sex determining mechanism but high incubation temperatures reverse genotypic males (ZZ) to phenotypic females. The W chromosome is thus unnecessary for female differentiation, which suggests that molecular mechanism directing sex determination is the result of the dosage balancing of a gene on the Z chromosome rather than the presence of a female determining gene on the W. That is, male differentiation requires two copies of a Z borne gene: the expression or gene activity is sufficient for male development only at optimal temperatures (Quinn et al.2007) [41].

c) Birds.

Reptiles represent the common ancestor for birds and mammals. From reptiles the evolution take two different pathways: one give rise to the branch of birds and the other one that culminate with the evolution of mammals.

Birds display a sex determining mechanism based on female heterogamety (ZZ/ZW). The sexual chromosomes Z and W have not relations with the mammalian X and Y; they could be evolved separately by different ancestral autosomic pairs and this could be a reason to explain the lack of knowledge about the localization of the sex determining gene in this class.

To this day there are two different theories under investigation. The first one considers the sex determination as the result of dosage of Z chromosomes in a quite similar scheme as described for *Drosophila* or *C.elegans*. One candidate gene could be *DMRT1* located on the Z chromosomes, escapes dosage compensation and is expressed specifically at gonadal level, and is thus capable of linking the numbers of Z chromosomes with gonadal differentiation. On the contrary, sex

determination could be the consequence of a female induction exerted by the presence of the W chromosome following the example of Y chromosome in eutherian mammals. Two different mechanisms are proposed to support this theory. The first one include the *FET1* gene which is localized exclusively in the W chromosome and it is expressed only in the female urogenital system. The second one refers to the homologues genes *WPKCI* and *ZPKCI* that are located respectively on the W and on the Z chromosome and to the different functional role resulting by dimerisation of their products (Smith and Sinclair,2004) [42].
The male genome produce an homodimer ZPKCI/ZPKCI acting as testis factor while the WPKCI/ZPKCI heterodimer prevent this effect.

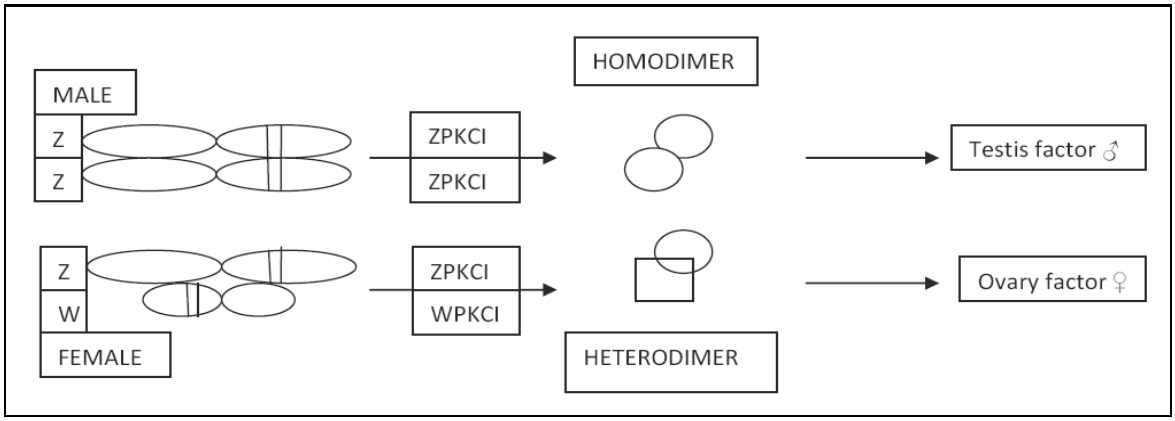

Figure 1.4: hypothetical role of ZPKCI in sexual determination. ZPKCI/ZPKCI homodimer stimulate a factor required for sexual determination in ZZ genetic males, whereas ZPKCI/WPKCI prevent the activation of the factor or stimulate directly ovarian differentiation in ZW genetic females.

To support this theory different combination of Z and W chromosomes caused by aneuploidy are investigated. So, organism with ZZZ present a testis but are not fertile, ZWW die early, but ZZW present a condition of intersexuality: these organism appear as female on hatching but slowly turn into males reaching the sexual maturity. These evidences could support the second theory.

d) Mammals.

Sex determination in mammals has been more extensively studied than in any other species, most probably due to its direct relevance to human physiology and pathophysiology. A large number of genes have already been described and many more are expected to be added in the process, since relevant research constantly reveals new players in the complex network of reactions related to sex determination. Even in the common, in bi-potential gonad, the expression of several genes is considered crucial for subsequent development and normal sexual dimorphism. These include, among others, *WT1*, *FtzF1/SF1* and *Lim1* (Angelopoulou,2005) [43]. Absence of any on these products at this stage, especially *WT1*, is inconsistent with further gonadal development and may also cause malformations. Sex determination in eutherian mammals is initiated by the *Sry* locus on the Y chromosome. *Sry* expression in the bi-potential, undifferentiated gonad directs the support cells precursors to differentiate as Sertoli cells, thus initiating the testis differentiation pathway. In

the absence of *Sry*, or if *Sry* is expressed at insufficient levels, the support cells precursors differentiate as granulosae cells, thus initiating the ovarian pathway.

Sry is a member of the large *Sox* HMG gene family postulated to have evolved from an ancestor of its paralogue on the Y chromosome, *Sox3* (Thompson 2004)[44]. This makes sense, as S*ox3* lies on the X chromosome and the Y chromosome is thought to have evolved from the X, and is consistent with the fact that birds and reptiles have neither a *Sry* gene nor a Y chromosome. Marsupials have the *Sr*y gene, but monotremes appear not to, which suggest, but certainly doesn't prove, that *Sry* evolved after the divergence of monotremes from the mammalian lineage.

The SRY protein is a transcription factor a SOX (*Sry*-like bOX) HMG (High Mobility Group) box type involved in the determination of cell fate and in the regulation of embryonic development (Koopman et al,1990) [45]. All SOX protein are transcription factors and interact with DNA to affect transcription (that is the generation of message RNA from the DNA template), either negatively or positively. SRY isn't a typical transcription factor, though, and all the players in mammalian sex determination pathway are not understood. Various elements such as WT1+KTS, GATA4, FOG2 are implicated in the regulation of *Sry*; *Sox9 i*s a downstream target of *Sry* and *Amh*, *Fgf9* and *Dax1* may be subsequent downstream targets involved in the cascade toward testis development. In the female embryo, the Y chromosome is not present and, therefore, *Sry* is not expressed. The genetic cascade regulating female reproductive system differentiation is not as extensively studied as in men, but *DAX1* (and its regulatory system, including genes such as *Wnt4* and *SF1*) is generally considered as a significant player in this process, which is how it came to acquire the rather simplistic description of the "antitestis gene" (Koopman et al.2001) [46].

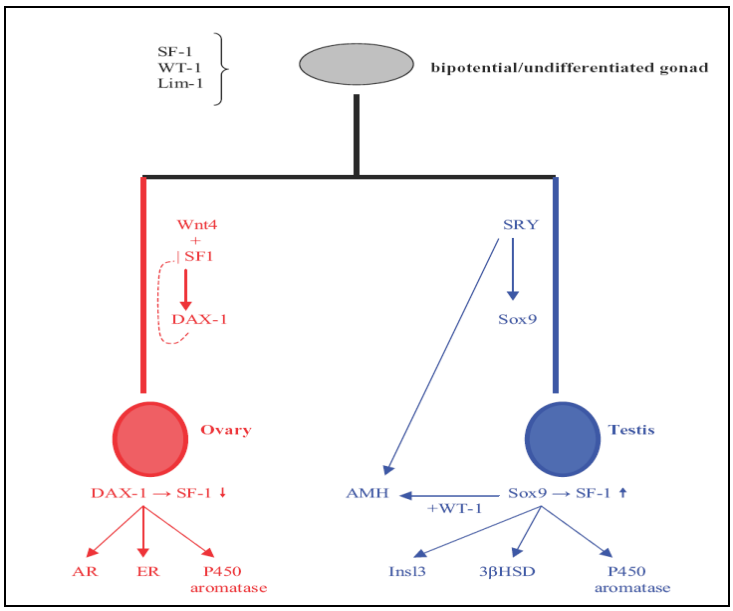

Figure 1.5: genetic model of sex determination in humans. The formation of the undifferentiated/bipotential gonad is controlled by several genes acting simultaneously, such as WT1, SF1 and Lim 1. Primary sex differentiation is based on the presence of the Y chromosome and its main sex-determining gene SRY. In this case SOX9, FtzF1/SF1 and AMH expression divert the gonad and the reproductive tracts towards the male phenotype. This differentiation process is regulated by several other genes, including DAX1, GATA4,FOXL2and, possibly, DMRT1 and 2. In females, SRY absence allows gonadal development towards female phenotype, mediated by genes such as DAX1, Wnt4 and SF1, resulting in aromatase upregulation. The exact role of Stra8 in this process remains unclear (from Manolakou, 2006) [47].

Two relatively recently described genes with a potential role in sex determination and differentiation are *DMRT1* and *Stra8* (stimulated by retinoic acid gene 8). The first has been already discussed previously as a conserved sex-related gene bearing a DM domain originally studied in nematodes. In humans, XY sex reversal in cases of 9p chromosome deletions have been attributed to impaired action of DMRT1 or its homologue DMRT2.
Stra8 on the other hand, is exclusively expressed in female germ cells and its presence signals their sexual gradual differentiation in an anterior to posterior direction. However, it has not yet been established whether the gene's product directly induces sex determinations towards the female pathway, or rather acts as a simple marker of this phenomenon, without active participation in the process.

e) Amphibians.

Amphibians employ a genetic mechanism of sex determination according to all available information on sex chromosomes or breeding tests. Several study have suggested that some amphibian species display ESD (Dournon et al,1990)[48]. In frogs, one species of *Bufo* and four species of *Rana* (Piquet,1930) [49] were examined. These studies all showed that the temperature of the rearing water can alter the sex ratios of tadpoles. In all of these studies, however, effects were obtained by exposure to temperatures that are not normally experienced by the species under study. In frogs 100% males were produce at high temperature (about 32°C), whereas 100% males or 100% females were produce in salamanders. A single study (Uchida,1937b) [50] showed that low temperatures (10°C) can produce 100% female also. When reared at temperatures within the ranges experienced naturally by the species under study, a 50:50 sex ratio was obtained in all of the studies cited above. The natural role of enviromental temperature in sex determination in reptiles or fishes is much more convincing, because effects have been observed at temperatures within ranges experienced by the species in the wild and effects have been shown in natural nests in turtles. Thus, given the lack of effects on sex ratio at appropriate temperatures for the species in the studies on amphibians, it is not likely that temperature is important in normal sex determination in amphibians. The reported enviromental influences are probably due to artifacts of the abnormally high temperatures at which the animals are reared. In addition, reptiles and fish displaying ESD typically lack sex chromosomes which is not the case in amphibians. Thus the available evidence suggest that sex determination in amphibian is under genetic control in natural condition (Wallace et al.1999) [51].
Certainly the presence of sex chromosomes is the clearest evidence that a species possesses a GSD. Sex chromosome can differ in morphology as result of loss of genetic material during the evolution or can be present no difference in shape and morphology if compared with the autosomic pairs. In the past, different techniques were adopted to examine preparations of metaphase spreads, in order to detect difference between the sex chromosomes. About 1500 species were investigated and only less than 4% of the amphibians examined cytologically posses morphologically distinguishable sex chromosomes. The inability to distinguish sex chromosomes suggest that a much smaller region may be involved in sex determination in amphibian sex

chromosomes relative to other vertebrates with morphologically distinct sex chromosomes (Hillis and Green,1990) [52].

Other experimental techniques that not require morphological identification of sex chromosomes have been used to identify the sex determining systems in amphibians. In most cases, experimental manipulation were used to obtain information regarding heterogamety. The natural condition of sex reversal in this class allows breeding tests between "sex-reversed" organisms to normal individuals, to establish which sex is heterogametic providing an indication of the mechanism of sex determination.

To induce the sex reversion, sex differentiation can be altered by treatment with exogenous steroid hormones or surgically . The effect of these treatments vary between species; the role of sex steroids in gonadal differentiation is not well known and also the sex steroids receptors have not been examined in amphibians.

Concerning the sex determining switch in this class no sex determining genes have been identified. The methodological approach used to investigate some putative genes is the comparative analysis of the conservative genes connected to sexual determination from invertebrate to high vertebrate throughout the evolution.

In addition to *Sry*, other genes such as *WT1, Fgf9, Dax1*, *Dmrt1* and *Sox9* are widely accepted to be involved in the sex determination in vertebrates. However, the roles of these genes during sex determination in amphibians is still unclear (Eggert 2004) [53].

Comparisons of *WT1* and *Fgf9* sequences between *R.rugosa* and other vertebrate species showed that both proteins have been highly conserved. The expression of these genes in the undifferentiated gonads began prior to sex determination but neither *WT1* nor *Fgf9* displayed a sexual dimorphism expression pattern at early developmental stages (Yamamura et al.2005) [54].

Concerning *Dmrt1*, the DM domain has a highly intertwined structure that chelates two zinc atoms, and makes specific DNA contacts predominantly in the minor groove (Zhu *et al.*, 2000) [55]. The DM domain gene family has some members with a conserved DNA-binding DM domain encoding putative transcription factors related to the sexual regulators *Doublesex* (DSX) from *Drosophila melanogaster* and *Male abnormal 3* (MAB-3) from *Caenorhabditis elegans* (Erdman and Burtis, 1993; Raymond *et al.*, 1998) [56,57]. *Doublesex* and *MAB-3* related transcription factor (*Dmrt*) gene is expressed in the frog *Rana rugosa* in the differentiating testes but is not detectable during ovarian differentiation (Shibata et al.2002) [58]. As a prelude to understanding the involvement of the *Dmrt* genes in sexual development in toads, the DM domain gene family of *B. gargarizans* was cloned and compared with other vertebrates (Chen and al.2007) [59]. *Dmrt* genes that are duplicated in *B. gargarizans* are present in mammals as single copies. For example, the human genome contains single copy of *DMRT3* (*DMRTA3*), whereas this gene is present in at least three copies in *B. gargarizans*. Gene duplication is a mechanism by which new gene functions may be acquired. The very recent duplication of the *Dmrt1* has apparently led to the formation of the master male-determining *DMY* gene in the medaka fish, and similar scenarios might have generated new paralogues of other *Dmrt* genes in different taxa (Mastuda *et al.*, 2002; Volff *et al.*, 2003) [60,61]. The *Dmrt* gene duplication in *B. gargarizans* may have been the result of such a process, unlike the situation in teleost fish, which may have been the result of the ancient

duplication of the whole genome. However, how these *Dmrt* genes function in the sexual development *B. gargarizans* still needs further experimental exploration.
Finally, the role of *Sox9* in the gonad will be discuss and examined later in section 3.5.

1.5) Reproduction strategies in the animal kingdom: costs and benefits.

In the animal kingdom are known two different strategies to perpetuate the species: asexual and sexual reproduction. Each one present costs and benefits associated with the typology of cellular division, number of progeny and in reposing at enviromental modifications.
Considering that each kind of reproduction is still now present we can argue that all of them are winning strategies from an evolutionistic point of view.
Asexual reproduction is a form of reproduction which does not involve meiosis, ploidy reduction and fertilization. This kinds of reproduction is typical of ancestral organisms that utilize only mitotic (equational) divisions in their reproduction strategy. Because asexual reproduction does not require the formation of gametes (as in gonochoristic animals) and bringing them together for fertilization, it occurs much faster than sexual reproduction and requires less energy. Asexual lineages can increase their numbers rapidly because all members can reproduce viable offspring. Other advantages connected to this kind of reproductive strategy include the ability to generate offspring without a partner in situations where the population density is low, reducing the chance of finding a mate, or during colonization of isolated habitats such as oceanic islands, where a single member of the species is enough to start a population.
A limitation related to asexual reproduction is that offspring are typically genetically similar to their parents; this genetic similarity may be beneficial if the genotype is well-suited to a stable environment, but disadvantageous if the environment is changing.
The second strategy is the sexual reproduction. The evolution of sex and sexual reproduction contains two related themes: its origin and its maintenance. However, since the hypotheses for the origins of sex are difficult to test experimentally, most current work has been focused on the maintenance of sexual reproduction.
It seems that a sexual cycle is maintained because it improves the quality of progeny (fitness), despite reducing the overall number of offspring. In order for sex to be evolutionarily advantageous, it must be associated with a significant increase in the fitness of offspring. One of the most widely accepted explanations for the advantage of sex lies in the creation of genetic variation. Another explanation is based on two molecular advantages. First is the advantage of recombinational DNA repair (promoted during meiosis because homologous chromosomes pair at that time), and second is the advantage of complementation (also known as hybrid vigor, heterosis or masking of mutations).
For the advantage due to creation of genetic variation, there are three possible reasons this might happen. First, sexual reproduction can bring together mutations that are beneficial into the same individual (sex aids in the spread of advantageous traits). Second, sex acts to bring together currently deleterious mutations to create severely unfit individuals that are then eliminated from the population (sex aids in the removal of deleterious genes). Last, sex creates new gene combinations that may be more fit than previously existing ones, or may simply lead to reduced competition among relatives.

For the advantage due to DNA repair, there is an immediate large benefit to removal of DNA damage by recombinational DNA repair during meiosis, since this removal allows greater survival of progeny with undamaged DNA. The advantage of complementation to each sexual partner is avoidance of the bad effects of their deleterious recessive genes in progeny by the masking effect of normal dominant genes contributed by the other partner.
In these organisms that display the sexual reproduction the genetic information is passed from parents to offspring via the germline, which segregates from the soma early in development and undergoes a complex developmental program to give rise to the adult gametes. Many aspects of germline development and germline proprieties such as cell fate, maintenance of cell identity, the migration of germ cells to the somatic gonadal primordium and proliferation of germ cells during development have been conserved throughout the animal kingdom from invertebrates to vertebrates.

1.6) Sexual differentiation in vertebrates: interpretative models.

Sexual differentiation is considered a conservative mechanism quite similar among vertebrates.
Starting from an hermaphrodite information the undifferentiated and bi-potential gonad is induced to follow one of the two alternative pathways reaching the development of a male or female sexuality.
The nature of the inductive signal is a controversy argument in developmental biology. Researchers supported different hypothesis that diverge in the attribution of the cause-effect relationships between somatic and germinal cells. These are the principals lineages:

- the classical and ancient conception identify the somatic cells as primary and causal inductors in sexual differentiation: in this model, germinal cells would represent undifferentiated and bi-potential passive targets that only under somatic inductions, exerted by different gonadal district, would be able to develop the two alternative phenotypes (symmetric model);
- in asymmetric model, germinal cells have an innate tendency to develop, without a somatic inductor, a “default” sexual phenotype. The alternative phenotype would be the result of a somatic inhibitory induction on a default program;

a) Symmetric model.

In the first year of the 1900 Witchi conduced a great number of observations on the gonadal organogenesis in amphibians by which he postulated a general developmental model of gonadal differentiation for the vertebrate classes.
In this hypothesis sex differentiation is seen as the result of sexualizing actions exercised by the somatic lineage on the germinal linage and the subsequently sexual development is the result of this cause-effect relationship.
In this first description Witchi, 1957 [62] illustrate the early embryonic gonadal architecture as an undifferentiated bisexual primordium with the capacity to differentiate into either sex; male or

female potentiality are represented by specific histological elements called cortex and medulla which have alternative roles in gonadogenesis.
The cortex, located in the distal portion, represents an extension of the somatopleuric epithelium and contain the germinal cells, whereas the medulla is composed by only somatic cells (derived from mesonephro).
By this description Witchi propose the model of "cortico-medullary inductors" in which these two different compartments could be able to produce different morphogenetic substances called "corticin" (produced by follicle cells) and "medullarin" (produced by interstitial cells).
The primary inductors are antigens and the corresponding anti-corticin and anti-medullarin suppressing the contrary inductor system, are responsible for the unisexual development of each individual.
Vannini, 1949 [63] purpose a different origin of the somatic cells: by the interrenal blastema and not by the mesonephic blastema. The significance of the difference between Witchi's and Vannini's model is that the gonadal primordial and the interrenal primordial (both steroid-producing tissues) share a common origin in Vannini's model, whereas the gonads share a common origin with the kidney primordial (non steroid-producing) in Witchi's model.
Lillie,1916 [64] developed a hormonal theory based on freemartin effect, in which he proposed that partial male development is imposed on the female twin in cattle by circulating sex hormones produced by her male co-twin. To account for the one way effect of sex reversal in freemartins Lillie pointed to evidence for the earlier appearance of steroidogenic cells in males, and concluded that differentiation of males occurs early enough to influence female development whereas development of female occurs too late to affect development of the male co-twin.
The experiments of Alfred Jost, 1970 [65] highlighted the existence of a discrete substance produced by testis implicated in the Müllerian ducts regression. His discovery of the Müllerian inhibitor, now called anti-Müllerian hormone (AMH) resolved the controversy surrounding the mechanism of somatic sex differentiation by proving that male characteristics must be imposed on the fetus by the testicular hormones testosterone and AMH, respectively responsible for the virilization of the Wolffian ducts, urogenital sinus and external genitalia and for the regression of Müllerian ducts. In the absence or in inactivity of these hormones, the fetus becomes a phenotypic female.

b) Asymmetric model.

The asymmetric model recognize at the germinal cells the innate tendency to develop, without a somatic input, a female phenotype. The bi-potent condition would be the result of a somatic inhibitory signal on a primary sexual development where the female development is the "default" pathway. The male phenotype would be the result of an inhibitory action on this general scheme.
The asymmetric model presuppose the existence of a homozygotic sex with spontaneous differentiation and a heterozygotic sex with secondary differentiation. By replacing the terms "homozigotic sex" and "heterozygotic sex" for "female" and "male" the representation of the differentiation process could be generalized to most vertebrate: self-differentiation of the gonadal organization, typical of "homogametic sex" would be inhibited and replaced by that which is

typical of "heterogametic sex" through interactions between germ cells and gonadal somatic cells, due to the expression of the H-Y or H-W serological specific antigens (Sxs).
In mammals, the Y chromosome is associated with the development of male sexual phenotype. The presence of the *Sry*, previously known as TDF (Testis-Determining Factor), is sufficient and necessary condition to induce a long cascade of events toward becoming male (Koopman et al.1991) [46].
Acting as molecular switch, *S*ry is considered the responsible for the sexual determination and its specific expression in the somatic cells of the gonadal primordium suggest that the somatic cells could be the inducer of the male sexual development supporting the asymmetric hypothesis (Bullejos et al.2001) [142].
Mutations or deletions of *Sry* are associated with the development of female phenotype in XY embryos. On the other hand, female mouse transgenic for *Sry* are induced to develop the testis with a typical mechanism of sex reverse.
Probably, the expression of *Sry* in XY embryos, could inhibit the female development and could induce the male development: if *Sry* is not express, the development follows the female pathway.
The female pathway would be the default mechanism.
At today, some evidence put in discussion the role of *Sry* as unique switch gene regulating the male development. For example, disruption of R-spondin 1 (RSPO1) is responsible for a human syndrome characterized by XX sex reversal, palmoplantar hyperkeratosis (PPK) and predisposition to skin carcinomas (SCC). *RSPO1* is required for ovary development since its disruption re-directs genetic female gonads to become testes in the absence of *Sry*. These genetic females are functional phenotypic males with no problems in fertility. However *RSPO1* is not required for testis differentiation, and XY individuals with homozygous mutation in *RSPO1* are male and fertile (Parma et al.) [66].
Sry would intervene in the complex cascade of events ending with the development of male phenotype but some question about the regulative mechanisms of sexual determination and differentiation are still quite unknown.

1.7) Development of the germ line in vertebrate.

In bilateral animals primordial germ cells (PGCs) originate outside of the gonads, to which they are transported later in development. At least two strategy to specify PGCs are present in vertebrates. Some species have a predetermined germ cell lineage that is specified by cytoplasmic germ cell determinants laid down in the oocyte and then differentially distributed to presumptive germ cells during embryogenesis. In other species, germ cells determination occurs later during development, and is not directly dependent on maternal molecules. In these species germ cell determination is governed by a regulative mode involving cell-cell interactions. Interestingly the amphibians shows either: the anuran use one of these mechanism and urodele the other. These difference are detectable also in amniotes: two different strategies are used to specify the PGCs.
In anuran, during oogenesis (for example in Xenopus) electron dense material known as germ plasm is syntetized and transported to the vegetal cortex through a specific transport mechanism involving cytoskeleton elements and in association with the mitochondrial cloud or Balbiani body

(King et al.,1999) [69]. Germ plasm is inherited by the most vegetal blastomeres of newly fertilized embryos, within the presumptive endoderm, and segregated into a few cells at the blastula stage (Huston and King,2000b) [70]. Those cells that inherit germ plasm will form the PGCs, and sister cells that don't inherit germ plasm become typical somatic endodermic cells. When PGCs reach the gonad, material resembling the electron-dense germinal granules of germ plasm, called nuage, accumulates in a perinuclear position within the PGCs.

In chicks the PGCs are derived from cells on the ventral surface of the area pellucid, a central region of the epiblast then migrate ventrally into the hypoblast and after become associated with the hypoblast move anteriorly to a region called germinal crescent within the area opaca. From germinal crescent the PGCs enter the embryonic circulation (Nieuwkoop and Sutasurya 1979) [71]. Later PGCs exit the circulation in the proximity of the gonad and are drawn to the developing genital ridges by chemotactic attraction (Kuwana et al. 1986) [72]. In chicks *Vasa* protein is present in germ cells during the entire cell cycle. *Vasa* is found in specific structures associated with the mitochondrial cloud in oocyte and is segregated into a few cells during cleavage. This suggest that germ cells are predetermined by inheriting maternal germ plasm.

In urodele, another mechanism is used to specifying germ cells. PGCs develop in the posterior-lateral plate mesoderm and are determined by zygotic influences, including posterior-ventral mesoderm inducing signals and subsequent cell interaction, not maternal molecules, as in anurans in a typical regulative germ cell specification.

The development of PGCs in mice occurs with parallelisms to the mechanism found in urodele. In mouse PGCs originate from cell located within the proximal epiblast and require signal from the bone morphogenetic proteins BMPs for their production (Ying et al,2001;Lawson et al. 1999) [73,74,75]. The PGC precursor migrate through the posterior primitive streak in association with extraembryonic mesoderm into the allantois, a posterior extraembryonic region (Ginsburg et al. 1990) [76]. Later they become associated with the hindgut and from there they move through the dorsal mesentery to the genital ridge (Anderson et al.,2000) [77]. A similar site of origin of PGCs has been described for several mammalian species. Germ plasm has not been found in early mouse embryos; on the contrary, any cell in the epibast of a mouse embryo can give rise to PGCs if placed within the proper signaling context (Tam e Zhou 1996, Yoshimizu et al 2001) [78,79] indicating that mouse PGCs are not predetermined. Nuage appears after specification when PGCs are in the hindgut (Eddy,1974) [80]. *mvh* expression (*mouse vasa homologue*) begin after colonization of the genital ridges by PGCs, in a very similar manner to the timing of *vasa* transcriptional activation in axolots.

1.8) Peculiarities of the germinal cells in anuran: nuage and germinal plasm.

Germ cells shows a distinctive propriety due to their unique role and function. In contrast to somatic cells and tissues which cease to exist in conjunction with the death of the organism, germinal cells are transmitted throughout the generations in a sort of immortal continuum.
Considering that the germinal cells develop in the adult each kind of tissue, they must remain pluripotent, but at the same time, they must become very specialized in differentiating the germ line (Kobayashi et L,1994) [82].
So that, the differentiation and the segregation of the germinal lineage from somatic lines in embryos are arguments of great interest to study both the propagation of species and the differentiation of a unique cell type.
It is well known that the special cytoplasm localized in eggs play an important role in the differentiation of certain cell types in many organisms (Davidson,1986) [81]; the continuity of germ cells depends on these factors that are inherited from each generation to the next, based in large part on the observation of distinctive cytoplasmatic structures in germ cells.
The germ cells contain a determinant of germ cell fate that is called germinal plasm. The germ plasm contains specific structural components such as P granules in *C. elegans*, polar granules in *Drosophila*, and germinal granules in amphibians such as *Xenopus laevis*. In *Xenopus* oocytes, the germ plasm is part of a specialized cytoplasmic organelle that is called mitochondrial cloud (MC) or Balbiani body. During oogenesis, fragments of mitochondrial cloud bearing the germ plasm migrate to the vegetal pole and become anchored at the vegetal cortex of the oocyte. Upon egg activation, the islands of germ plasm coalesce into larger aggregates, and during embryogenesis, they segregate to the vegetal blastomeres. Subsequently the descendants of vegetal blastomeres that contain germ plasm become the primordial germ cells.
Germ plasm is the specific material of cytoplasm containing determinants of a germ cell line that provide maintenance of this line and development of gametes. The segregation of germ and somatic lines in embryos is one of the fundamental events in ontogenesis.
Nuage material is been found in different stage of development of the germinal cells: from PGCs (Primordial Germ Cells) in embryos to gametes in adult gonads.
In many animals, nuage has been shown to contain RNA and proteins and is widely believed to carry the determinants of the germ line while germ cell differentiation depends on a special region of cytoplasm in the embryo called germ plasm, which closely resembles nuage in morphology and ultrastucture.
In *Drosophila*, *C.elegans* and *Xenopus*, germinal plasm is assembled from germline specific factors that are deposited in the egg during oogenesis: it is well localized and determinates where the PGCs will form.
Germ plasm is distinguished by the presence of organelles termed germinal granules. Like nuage, germinal granules are not surrounded by a membrane, display of fibrous particles and are in association with mitochondria, many RNA and proteins.
The germinal plasm is present in the endoplasm of post vitellogenic oocyte and during the first steps of the embryogenesis.

One of the unresolved question on regards the relationship between nuage and germinal plasm during the differentiation. These structures share a lot of characteristics as morphology, presence of mitochondria, RNA and proteins, and are localized in the germinal line: these observation suggest that nuage and germinal plasm could represent the same material in different forms in which nuage could represent a precursor of the germinal granules.
Support for this idea is the presence of Vasa protein in both germinal granules and nuage in Drosophila and in embryonic and adult germinal cells in *Xenopus*, indicating a direct link between this structures.
However mammals don't contain a germ plasm, even thought germ cell-specific nuage has been well characterized in various species.

1.9) Sex differentiation and gonadal development in amphibians.

Amphibians are gonochoristic animals; the gonads of male or females larvae are paired structures that develop from sexually undifferentiated primordial gonads formed by somatic and germinal cells located near the presumptive kidneys.
The undifferentiated and bi-potential primordial gonad is composed of medullary and cortical regions covered by a basal lamina. A lack of ultrastructural differences between medullary and cortical cells of the primordial gonad indicates a cortical origin of the medullary tissue. In later stages, the mesogonium is invaded by blood vessels, nerves, and opisthonephric interstitial tissue. The primordial germ cells are located in the cortical region of the undifferentiated gonad forming a primitive germinal epithelium together with surrounding pre-follicular cells. The PGCs commonly become smaller after they enter the genital ridge in association with proliferation and digestion of yolk platelets.
Gonadal sex differentiation coincides with metamorphic climax in almost all the species of amphibians. Female and male gonads are distinguishable by the number and size of germ cells and the amount of medullary tissue. In female the cortex develops and growth leaving a hole (ovarian vescicle) which is observable upon histological analysis. The cell in the cortex become large follicle and oocyte can be observed during early stage of differentiation.
Larval ovaries are larger than testes and have a slightly irregular outline. They usually consist of medullary and cortical tissue in which the proliferating gonial cells from several groups of oogonia and oocytes. Oogonia measure 17-25 um in diameter and cytologically resemble PGCs except for the lack of pigment granules and abundant ER. The transition from primary to secondary oogonia (primary oocytes) is characterized by a dark cytoplasm and a change from lobed to round nuclei. Oogonia and primary oocytes contain germ plasm. Prophase and primary oocytes is initiated in larvae prior to metamorphosis. At metamorphic climax larval ovaries exhibit oogonia and leptotene, zygotene, pachytene and diplotene oocytes. Near the end of leptotene, oogonial chromosomes attach to the inner surface of the nuclear membrane (bouquet arrangement) at a side adjacent the juxtanuclear mitochondrial aggregate, which frequently forms a cap over one end of the nucleus. The zygotene stage is characterized by the formation of short, axial chromosomes. Paired homologues chromosomes form synaptonemal complex and synapsis is

completed in pachytene. The cytoplasm and nucleus enlarge in diplotenic oocytes, synaptonemal complexes are absent, and the chromosomes are in lampbrush stage.
During early diplotene, the Balbiani body (mitochondrial clouds) is formed by condensation of the juxtanuclear mitochondrial aggregate. In the late diplotene oocyte, dispersed germ plasm is stored in the cortex of the oocyte.
In most anuran larvae, testicular differentiation starts at pre-metamorphosis stage; the primitive germinal epithelium disintegrates to a simple peritoneal surface epithelium. The male gonadal cells or spermatogonia together with their follicular cells, are scattered throughout the compact medullary tissue, which becomes mixed with interstitial mesenchymal cells. In the center of the medulla a network of tubules become increasingly distinct and finally differentiates into seminiferous tubules. The gonads in males have smaller densely packed cells.

1.10) Architecture of the genital ridge in bufonids.

The macroscopic morphology of the genital ridge in bufonids present the distinction in two different compartments: the cephalic tract represented by the Bidder's organ, and the caudal tract corresponding to the functional gonad. Bidder's organ develop as a rudimental ovary both in males and in females, whereas the gonad develops with a cephalic-caudal gradient following the alternative pathways.

a) Bidder's organ.

The natural and peculiar condition in bufonids is the most compelling case for hermaphroditism in amphibians. This rudimental character, common in bufonids, is associated with the presence of the Bidder's organ. In bufonids, both males and females posses a pair of rudimentary ovaries called Bidder's organs, which are located at the anterior portion of the gonadal primordium. The Bidder's organ was first described as fat (Roesel,1758) and then as the primordium of the testis (Ratchke,1825). Bidder (1846), from whom the organ was named, argued a function as accessory structure involved in male gametogenesis. Von Wittich (1853) was the first to suggest that the Bidder's organ was a rudimentary ovary developing in both males and females, and then histological data supported his hypothesis. The Bidder's organ have the potentiality to develop into a functional ovary able to produce eggs in males specimens after removing testes by surgically castration (Ponse,1923,1930) [83,84].
Bidder's organ develop from at the anterior mesogonial portion of the genital ridge. Because neither corticomedullary structure nor primitive gonadal cavities develop, Bidder's organ consists of a cortical layer of flattened cells, peripherally located gonia, a few oocytes, and large auxocyte-like cells that partially degenerate postmetamorphically. In Bidder's organ, oogenesis is an abortive process because the oocytes fail to reach vitellogenesis and undergo a degenerative fate.
The ovarian differentiation of Bidder's organ begins during larval stages regardless of sex genotype, while the posterior gonadal primordium starts to differentiate into a proper ovary or testis only at the end of metamorphosis.
The normal organogenesis of this abortive ovary is characterized by a differential distribution of PGCs migrating in the genital ridges according to a cephalo-caudal gradient. The proliferative rate

of bidderian GCs is chronologically more advanced than the gonadal GCs. Furthermore, during larval life, two subsequent oogenetic waves occur in the bidderian region: the first one during stage IV9 the second one at the complement of metamorphosis (stage IV17).
In females organisms, the second bidderian oogenetic process extends as far as the gonad which represent the first ovarian oogenetic wave.

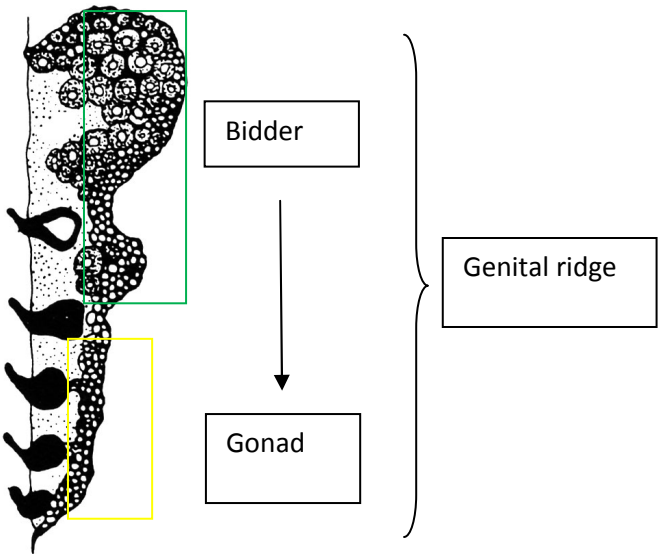

Figure 1.6: **genital ridge of *B.bufo* specimen.** Genital ridge organization in *Bufo* larvae according to a cephalic-caudal gradient of differentiation (from Witchi,1956).

According to Cambar e Gipoloux staging system, the classical description of Bidder's organ, starts from stage IV4 after the migration complement of PGCs from the dorsal mesentery to the genital ridge. From the analysis of transversal sections at stage IV4, Bidder's organ present an oval shape and an organization in a unique compartment corresponding to the Witchi's cortex and termed peripheral compartment (PC). PC is composed by external somatic cells in continuity with the mesogonial portion delimiting the organ, and germinal cells (oogonia).
Reaching stage IV9, the architecture of the Bidder's organ presents a tripartite subdivision that replace the ancient and classical descriptions of bipartite organ (medulla, cortex). These compartments are represented by the medulla and two branches (peripheral compartment and follicular compartment) arising from the division of the cortex. The most external region consists of a peripheral compartment (corresponding at the unique compartment of stage IV4), containing small diplotenic oocytes and somatic cells.
The peripheral compartment assumes the meaning of germinative epithelium: it contains the germ cells that give rise at the two bidderian oogentic waves produced, at first, by mitotic multiplication and later by meiotic multiplications (Falconi et al.2001) [141]. Germinal cells in mitotic and meiotic phases are described as:
-oogonia: are diploid cells, localized in the peripheral compartment, whit lobed nuclei, isolated by a wrapper of somatic cells;
-mitotic oogonia: are diploid cells, localized in the peripheral compartment, composed by two or more oogonia that could be in the same stage of maturation (synchronous nests),or in different stages (asynchronous nests), whit lobed nuclei;

-pre-meiotic oocytes: are localized in the peripheral compartment, grouped in asynchronous nests, with round nucleus. These germ cells are defined as oocytes because retained at the beginning of the meiotic prophase I;
-synaptic oocytes: are localized in the peripheral compartment in asynchronous nests (with mitotic oogonia or pre-meiotic oocytes) or in synchronous nests, with synaptonemals complex in their nuclei.
Entering into long diplotenic phase oocytes tend to isolate, each one wrapped by somatic cells, and begin to escape from the peripheral compartment towards the primary cavity to the follicular compartment. The intermediate or follicular compartment (FC), more central, is constituted by follicles of diplotenic oocytes of the first bidderian wave. The central compartment, or medulla, is composed exclusively by somatic cells that are not distinguishable from those belonging to other regions of the organ. By microscopic analysis, the medulla appears different in size, being relatively abundant in some sections and composed of a limited number of cells in others. Furthermore, the medulla changes in size during development: it is absent in the stadium IV4, clearly visible starting from the stage IV9 and then regress completely reaching metamorphosis (stage IV17). The medulla of Bidder's organ not present a cavity, unlike what happens to the levels of ovarian genital body in *B.bufo*.
Reaching stage IV17 Bidder's organ present a bipartite structure composed by the peripheral and follicular compartment. While PC not underline differences in organization respect stage IV9 the follicular compartment increase its volume. FC is a typical ovarian structure, which consists of diplotenic oocytes in the second period of growth (auxocytosis) that are leaked by the peripheral compartment to the primary cavity, wrapped individually by a follicle of somatic cells (follicular cells). This mechanism takes place with the formation in the peripheral tissue of a sort of hernia within the primary cavity. The growth of the oocyte, during the auxocitosis period, induce the cell to "fall in" the primary cavity with an almost passive mechanism. Somatic cells that accompany the oocyte in this movement seem to be derived all from the peripheral compartment: there is not an interruption in the peripheral compartment to allow at the somatic medullar cells to participate in the construction of the follicle. Moreover, the discovery of mitosis in somatic follicular cells confirms the hypothesis that the growth of follicular envelope, which is necessary to follow the great increase in the volume of the diplotenic oocyte, is due to the division of cells that already are part of, rather that the contribution of other outer somatic cells.
Concerning the putative synchrony of mitotic and meiotic divisions regarding germinal cell of the same nest, it seem obviously, that at least the meiotic division do not occur simultaneously (gonia and oocytes in the same nest). The fact could be related with the establishment of a reserve of proto-gonia to support the two bidderian oogenetic waves that continue in both sexes even in adults. Since the colonization of gonadal primordium by PGCs deriving from extra-gonadal territories (embryonic endoderm, Blackler,1966) [87] occurs only in early stages of development (Delbos et al.,1982a) [88] and does not repeat itself, it is clear that a proportion of primordial germ cells that are in the germinal epithelium must retain "stem cells" to form a reserve of germinal elements. This could be the meaning of gonia "retarders" nests found together with oocytes after maturation in most advanced differentiation.

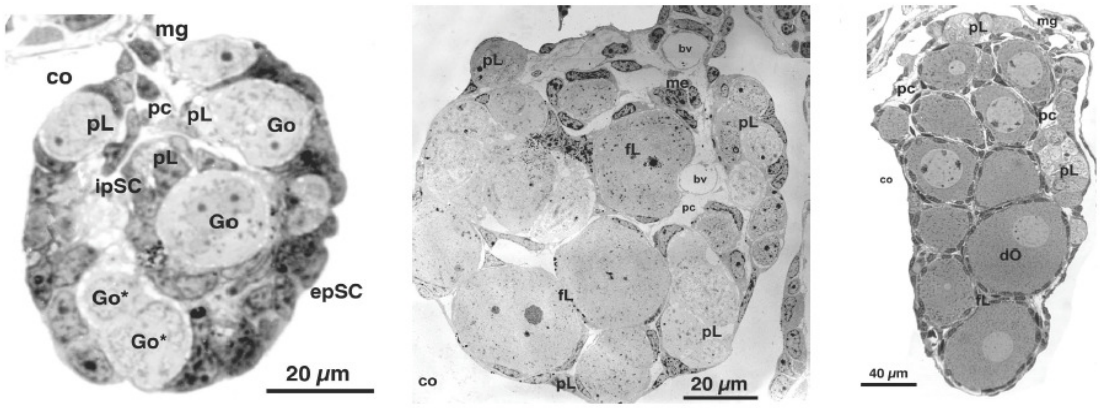

Figure 1.7: Bidder development during stage IV4, IV9,IV17. These pictures show the principal developmental stages of Bidder's organ. mg=mesogonial portion, pc=primary cavity, Go=gonia , epSC= external somatic cells, ipSC= internal somatic cells, co=celomic cavity, pL= peripheral compartment, fL= follicular compartment, dO=diplotenic oocyte, bv=blood vessel, Go*=mitotic gonia (from Dalpiaz,1998) [140].

Stage	IV4	IV9	IV17
PC	oogonia	oognia, small oocytes	oogonia, small oocyte, small diplotenic oocytes
FC		diplotenic oocytes	diplotenic oocytes (auxocitosis)
CC		somatic cells	

Table 1.1: principal events during developmental stages in Bidder's differentiation: Differentiation of Bidder's organ architecture from early stages of development to the complement of metamorphosis. PC= peripheral compartment, FC= follicular compartment, CC= central compartment.

***b) Gonads*.**

During larval life the tract of the genital body behind the Bidder's organ maintains an undifferentiated condition with germ cells localized in the cortex (gonia or mitotic gonia) and presence of compact medulla at the center of the gonad.

B.bufo gonads of tadpoles differentiates only few days before the complement of metamorphosis: in genetic females we observed a caudal extension of the Bidder's second oogenetic wave (first ovarian wave) with atrophy of the medullar tissue that is reduced to one layer coating of the ovarian cavity.

In genetic males, the gonad inverted the ovarian orientation with a reduction of cortical territory and germ cells migration into medullar tissue, where germ cells develop as spermatogonia. Gonadal sex differentiation is nearly complete during the first month of life post-metamorphic.

During gonadal development we can describe different stages of interest. At stage IV4 the undifferentiated and bi-potential gonad contains PGCs and an envelope of somatic cells distributed in a unique compartment. This architecture is maintained until stage IV9.

From stage IV12 begin to diverge two different morphological typologies that represent the alternative developmental pathways.

Females: at stage IV12 the gonad present the typical subdivision in two different compartments: a peripheral one (cortex) containing somatic and germinal cells delimited by a basal lamina towards a primary cavity, and a central one (medulla) formed by only somatic cells ad delimited by another basal lamina. During stage IV13 the gonadal volume increase as consequence of mitotic and

meiotic divisions of the peripheral compartment, whereas inside medullar compartment begin to form the incipient ovarian cavity. At the complement of metamorphosis, at stage IV17, the definitive architecture is quite similar to that observed at stage IV13: a bipartite structure formed by a peripheral germinal layer with the medullar somatic cells reduced at a mono layer delimiting the ovarian cavity .

At the complement of metamorphosis female gonadal architecture resumed the ancient undifferentiated morphology.

Males: at stage IV12, as in females organisms, the gonad is divided into an external cortex and a central medulla. The first evidence in male gonadal differentiation begin during stage IV13 with a great reorganization in morphology. This is realized by the dissolution of the basal laminas. As a consequence of these dissolution in gonadal compartment, germ cells migrate into the medulla and interact with the medullar somatic cells. Is interesting underline that few germ cells don't reach the medulla staying in the cortex. This fact could be interpreted as a sort of "reserve" of germinal elements. The germinal cells stop the migration at the complement of stage IV14 stage. During this period mitotic divisions are stopped. Reaching stage IV17 the morphology is similar.

	IV4	IV9	IV12	IV13	IIV17
Undifferentiated	Unique compartment	Unique compartment			
Females			Cortex, Medulla	Germ clls in the cortex, ovarian cavity primordium	Germinal cells in the cortex
Males			Cortex, Medulla	Centripetal migration of germ cell inside the medulla	Germinal cells inside the medulla

Table 1.2: Principal steps in gonadal development during embryonic life of *B.bufo* specimen.

IV4 size = 13,5 mm

IV9 size = 25mm

IV12 size = 28 mm

IV13 size = 24 mm

IV17 size = 10mm

Figure 1.8: morphological changes in *B.bufo* larvae during developmental stages.

2.PURPOSE OF THE RESEARCH

Amphibians represent an excellent models to study the mechanisms of sexual determination and sexual differentiation for a number of reasons. Genetic sex-determining systems and sex chromosomes have evolved a number of times and gonadal differentiation, although under genetic control, is responsive to experimental manipulations. In addition, the natural and peculiar condition in bufonids is the most compelling case for hermaphroditism in amphibians. This rudimental character, is associated with the presence of the Bidder's organ: both males and females posses a pair of rudimentary ovaries (Bidder's organs), which are located at the anterior portion of the germinal ridge.

Furthermore, as laboratory animals, amphibians are ideal because female produce a great number of eggs large in size. A great number of tadpoles can be treated in experimental manipulation because larvae are typically aquatic and readily adsorb hormones added to the rearing water.

On the other hand, most amphibians lack morphologically distinguishable sex chromosomes and no sex determining genes are known.

The general purpose of this investigation is referred to the analysis of the different competence of somatic and germinal districts elements during the alternative pathways of sexual differentiation. The bi-potential condition of the germinal cells, culminating into oogenesis or spermatogenesis, could be referred as a previous basilar condition that anticipates the sexualizing actions exerted by the gonadal somatic elements or as the result of an inhibitory effect exerted by the somatic cells on a female predetermined developmental pathway.

To investigate the interactions between germinal and somatic cells during the alternative pathways, we have to discriminate the sex of the organisms at early stages of development by detecting sex-markers or by overriding the genotype of samples inducing a unique sexual phenotype. In this purpose we have tried different experimental techniques.

Section 3.3 examine the sex determining mechanism by using bio-molecular approaches. We utilized RAPD's primer because is a simple and fast method to detect polymorphisms especially if genetic information are unknown. Another approach we used is the analysis of AAT gene (sex linked in *R.rugosa*) in *B.bufo* adult specimens .

Section 3.4 investigate both epigenetic factors that are able to override genotypic sex determination in amphibians such as hormonal treatments, and both sexual inversion in *B.bufo* males in order to produce an unisexual offspring as consequence of mating between "inverted" and "normal" males.

At last, in section 3.5 are analyzed the germ-soma interactions during the differentiation of the alternative phenotypes by investigating the gonadal morphology utilizing both confocal laser scanning microscopy with antibody anti-α-tubulin, and confocal laser scanning microscopy with antibody anti-Vasa protein.

To understand the putative role of *Sox9* as sex marker in male development, we tried wmISH (wholemount in situ hibrydation) to detect different expression patterns.

3.MATERIALS AND METHODS

3.1 Samples.

Bufo bufo specimen (eggs, tadpoles and adults) are collected from the sedentary population living in the artificial pond of the Botanical Gardens of the University of Bologna. Organisms were subsequently transferred to the laboratories of the Department of Evolutionistic and Experimental Biology (BES).
Eggs and tadpoles were reared in containers with dechlorinated water and fed with boiled spinach until the end of their embryonic life.
Adult organism were fed with different kinds of insects larvae.

3.2 Staging of tadpoles.

Staging is the recognition of certain morphological landmarks that appear useful in comparing the sequence of events in a developmental continuum. With the use of a staging system we can refer the observation to a particular period during the morphological development, starting from the early embryo (after fertilization) and ending with the complement of metamorphosis.
It is very important to correlate the observation at the developmental stage in order to compare events in different organisms. The developmental stages of *B.bufo* tadpoles are referred to Cambar-Gipouloux (1956) [89] staging system.

C-G	G	%
I1	3	0,1
I2	4	0,2
I3	5	0,3
I4	6	0,4
I5	7	0,6
I6	8	1
I7	9	1,2
I8	9	1,6
I9	10	1,9

C-G	G	%
II1	10	2,3
II2	10	2,6
II3	11	2,8
II4	11	3,0
II5	11	3,4
II6	12	3,9
II7	12	4,2
II8	13	4,3
II9	14	4,6
II10	15	4,8
II11	16	4,9
II12	16	5,3

C-G	G	%
III1	17	5,9
III2	18	7,1
III3	18	7,9
III4	18	9,1
III5		10,8
III6	20	12,0
III7	21	13,4
III8	23	15,0
III9	24	17,1
III10		18,0

C-G	G	%
IV1	26	19,1
IV2	28	22,8
IV3		25,0
IV4	**29**	**31,3**
IV5	30	39,6
IV6	31	43,8
IV7		52,1
IV8	32	56,3
IV9	**34**	**64,6**
IV10	35	68,8
IV11	40	77,1
IV12		**81,3**
IV13	**41**	**85,4**
IV14		87,5
IV15	42	88,5
IV16	44	94,8
IV17	**46**	**100,0**

Table 3.1:developmental stages. Comparision between Cambar-Gipoloux (C-G) staging system (1956) and Gosner (G) staging system (1960) and time (%) to reach metamorphosis (express as percentage to the complement of metamorphosis).

3.3) Sexual determination: bio-molecular approaches.

a) Introduction.

Section 3.3 described the different methodological approaches we utilized with the purpose to found new evidences in the complex mechanism of sexual determination in amphibian.
Although *Bufo bufo* display a ZZ/ZW type chromosomal sex determination system, the molecular mechanisms of sex determination and differentiation remain unclear for this species. Sexual chromosomes are homomorphs, moreover no sex determining gene have been described in amphibians. Genes such as *WT1, Fgf9, Dax1, Dmrt1* and *Sox9* are widely accepted to be involved in the sex determination in vertebrates, however, the roles of these genes during sexual determination in amphibians is still unclear. Also, unlike in mammals, gene-dosage compensation does not occur in amphibians. Bar bodies or other mechanisms of deactivation of the homogametic sex chromosome have not been observed.
For these reasons the purpose of bio-molecular approaches we utilized, is focused on the research of sex markers in order to discriminate male and female genotypes. Utilizing a sex marker, the study of the alternative gonadal developmental pathways could be investigated during the entire developmental pathway staring from the undifferentiated gonadal condition.
The strategies we utilized, are founded on the detection of positive or negative polymorphisms sex associated.
At first we tried to detect sex-linked polymorphic bands in males and females specimens utilizing RAPDs primers because it is a fast and relatively simple technique for marker identification in animals, especially in species for witch DNA sequence information are not readily available.
We tried also in to amplify by PCR in *B.bufo* a partial sequence of the AAT gene, exon 2, by specific to verify if the alternative allelic sequences on Z and W chromosomes as in *R.rugosa* is manteined.

b) RAPD (Random Amplified Polymorphic DNA).

Random Amplified Polymorphic DNA (RAPD) markers have been described as a simple and easy method to detect polymorphisms based on the amplification of random DNA sequence with single primers of arbitrary nucleotide sequence and length (Welsh and McClelland 1990; Williams et al. 1990) [91,90]. Genomic DNA is subjected to the polymerase chain reaction (PCR), adding only a single short oligonucleotide of random sequence in each PCR reaction. Typically, in standard RAPD analysis, the sequence of each primer should respect few basilar principles. Generally is a short (10-mer) arbitrary oligonucleotide with a 50%-70% composition in Guanine plus Cytosine , with Guanine or Cytosine in 3', with no self-complementary extremity.
Unlike traditional PCR analysis, RAPD does not require any specific knowledge of the DNA sequence of the target organism: the identical 10-mer primers will or will not amplify a segment of DNA, depending on the distance of positions that are complementary to the primers' sequence. A DNA amplification product is generated for each genomic region that happens to be flanked by a pair of priming sites, in the opposite orientation, which are 5 kpb of each other; no fragments are produced if primers annealed too far apart or 3' ends of the primers are not facing each other.

The polymorphisms detection is due to the presence or absence of bands and may be caused by failure to prime a site in some individuals because of nucleotide sequence differs or by insertions or deletions in the fragment between two priming site.
The method has considerable advantages because is fast, can be used with limited amount of DNA and can detect polymorphism in any kind of sequences. Among the multiple application of RAPDs are their use in population genetic (Haig et al.1994) [92], taxonomy (Chapco et al.1992) [93], determination of paternity (Lewis and Snow 1992)[94] and mapping (Michelmore et al. 1991) [95].
The RAPD method present also some limitation. All RAPDs markers are dominant, it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous (1 copy) or homozygous (2 copies). RAPDs fragments are produced by PCR reactions, therefore the quality and concentration of template DNA, PCR components (such as [$MgCl_2$] or polymerase), and the PCR cycling conditions may greatly influence the outcome (Williams et al.1990, Arnold et al. 1991) [90,96]. Thus, the RAPD technique is notoriously laboratory dependent and needs carefully developed laboratory protocols to be reproducible. Mismatches between the primer and the template may result in the total absence of PCR product as well as in a merely decreased amount of the product.
Many bands of mixed intensity are generated with each primer and artifactual bands may arise from hetero-duplex formation between amplified products (Ayliffe et al.1994) [97] or from other secondary artifacts (Hadrys et al.1992) [98]. Thus, the RAPD results can be difficult to interpret.

c) Genomic DNA extraction.

Genomic DNA were extracted from livers (four males=M,1,2,3 and three females= F,4,5) of *Bufo bufo* adult specimens in agreement with the Qiagen QIAquick DNA blood and tissue extraction kit protocol. DNA concentration was quantified with a spectrophotometer and further checked in a minigel.

d) Primers.

Oligo Name	Oligo sequence (5' to 3')	Scale of synthesis	Purif.	Oligo Name	Oligo sequence (5' to 3')	Scale of synthesis	Purif.
OPA-08	GTGACGTAGG	0.05	DESALT	OPK-06	CACCTTTCCC	0.05	DESALT
OPA-11	CAATCGCCGT	0.05	DESALT	OPK-09	CCCTACCGAC	0.05	DESALT
OPA-12	TCGGCGATAG	0.05	DESALT	OPK-14	CCCGCTACAC	0.05	DESALT
OPA-19	CAAACGTCGG	0.05	DESALT	OPK-18	CCTAGTCGAG	0.05	DESALT
OPB-19	ACCCCCGAAG	0.05	DESALT	OPM-02	ACAACGCCTC	0.05	DESALT
OPC-07	GTCCCGACGA	0.05	DESALT	OPM-04	GGCGGTTGTC	0.05	DESALT
OPC-17	TTCCCCCCAG	0.05	DESALT	OPM-14	AGGGTCGTTC	0.05	DESALT
OPD-01	ACCGCGAAGG	0.05	DESALT	OPM-17	TCAGTCCGGG	0.05	DESALT
OPD-15	CATCCGTGCT	0.05	DESALT	OPN-09	TGCCGGCTTG	0.05	DESALT
OPD-16	AGGGCGTAAG	0.05	DESALT	OPN-14	TCGTGCGGGT	0.05	DESALT
OPF-11	TTGGTACCCC	0.05	DESALT	OPO-01	GGCACGTAAG	0.05	DESALT
OPG-07	GAACCTGCGG	0.05	DESALT	OPO-04	AAGTCCGCTC	0.05	DESALT
OPH-06	ACGCATCGCA	0.05	DESALT	OPO-16	TCGGCGGTTC	0.05	DESALT
OPH-07	CTGCATCGTG	0.05	DESALT	OPO-18	CTCGCTATCC	0.05	DESALT
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OPI-04	CCGCCTAGTC	0.05	DESALT	OPP-10	TCCCGCCTAC	0.05	DESALT
OPI-14	TGACGGCGGT	0.05	DESALT	OPP-11	AACGCGTCGG	0.05	DESALT
OPJ-01	CCCGGCATAA	0.05	DESALT	OPP-12	AAGGGCGAGT	0.05	DESALT
OPJ-03	TCTCCGCTTG	0.05	DESALT	OPP-17	TGACCCGCCT	0.05	DESALT
ABI-04	GGACTGGAGT	0.05	DESALT	ABI-01	GTTTCGCTCC	0.05	DESALT

Table 3.2:primers. List of primers used in RAPDs.

e) Amplification conditions.

Amplification reactions were performed in 500mM KCl,150mM Tris-HCl (GeneAmp 10X PCR Gold Buffer, Applied Biosystems), 3mM $MgCl_2$, 0.25 mM dNTP Set (Fermentas) , 0.6 µM primer (Sigma Genosys), 2 ng template DNA and 1.5 U Taq polymerase (AmpliTaq Gold, Applied Biosystems) in a final volume of 25 µl.
DNA amplification was performed in a thermal cycler programmed as follows: 94°C for 5 minutes followed by 45 cycles consisting of 94°C for 1 min, (Ta=Tm-5°C) for 1 min and 72°C for 2 min. A final extension was carried out for 7 min. One negative control (absence of template DNA) was performed for each set of amplifications. PCR products were separated by electrophoresis in 1,4% agarose gel in 1X TBE buffer. Molecular sizes of the amplification products were estimated by using a 0,1-10,0 Kb DNA ladder. Gel were stained in ethidium bromide solution and observed under UV light.
The usual routine precautions were adopted when performing PCR reactions. In particular, all reagents and equipment were autoclaved, oligonucleotides were resuspended in MilliQ sterile water and aliquoted in separate DNA free laboratory tubes, reagents were aliquoted in separate DNA free laboratory tubes, gloves were worn when handling PCR components or preparing PCR reactions.

f) Band patterns.

As observed by other authors (Williams et al. 1990, Haig et al. 1994) [90,92] negative controls occasionally produced bands that are usually faint. However, bands in negative controls were always different from bands in the amplification reactions.
Bands were between 200 and 3000 bp in size. RAPD profiles included bands with different degrees of sharpness and intensity.
Only few primers detected polymorphisms between male and female, but they are not been reproducible.

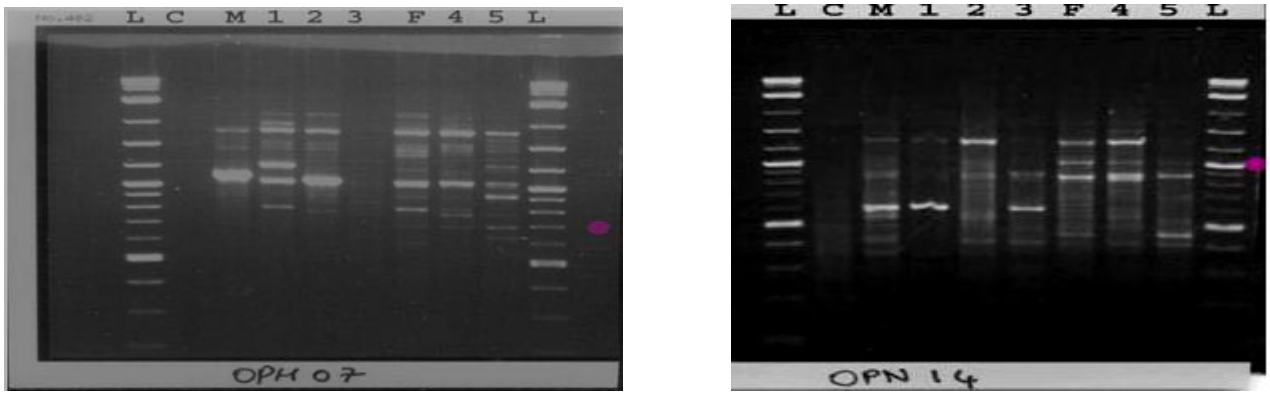

Figure 3.1: **RAPD profiles using primer OPH7,OPN14**. Putative polymorphic bands are near the violet circle in female specimen. L=DNA ladder; C=control; M,1,2,3= males; F,4,5=females.

RAPDs primers have not provide the expected results; the limitation are related to the low percentage of detected polymorphisms and to the not reproducibility of the data.
The reproducibility is a limitation associated with the technique itself as reviewed by other authors (Perez et al.1998) [99]: little difference in amount of DNA, temperature of annealing and reagents can determine different bands patterns. For this reason we have to improve our protocol.
Concerning the low percentage of polymorphism, the fact is partially due to a low number of tested primers (only 40). Increasing the number of primers more polymorphism are expected. Another problem related to the polymorphism is connected to the fact that polymorphic bands between males and females organisms could be associate only with phenotypic differences and not with sex-markers. Focusing the research on genomic DNA extracted by a single couple of parental organisms, and by their offspring at the complement of metamorphosis we could increase the percentage of sex linked polymorphisms.

g) AAT (ADP/ATP translocase).

The ADP/ATP translocase gene (AAT), also known as the adenine nucleotide translocase (ANT) or ADP/ATP carrier, has an important role in ADP and ATP transport on the inner mitochondrial membrane and in forming the mitochondrial permeability transition pore (Kokoszka et al.2004) [100]. The structure is very conserved, however the AAT gene is known to exist as a multiple copies in most species; for example humans have 3 genes isoforms termed respectively *Ant1, Ant2* (X-linked) and *Ant3*.

h) Sexing by PCR-RFLP in Rana rugosa.

The frog *R.rugosa* is found in four different countries in the area surrounding the Sea of Japan: China, North and South Korea and Japan (Okada, 1930). The Japanese population are divides into four genetic forms that inhabit the four different geographical regions. These forms differ in the heterogametic sex determination and sex chromosome differentiation. One of the four forms, termed ZW form (North-west Japan), has female heterogamety sex determination with differentiated sex chromosomes (number 7), whereas the other three forms named Kanto, West Japan and XY forms, have male heterogamety sex determination. Only the XY form (central Japan) has differentiated sex chromosomes (number 7) whereas the other two population lack morphologically distinguishable sex chromosomes (Miura).
Miura *et al*. (1998) [101] isolated the sex-linked gene, ADP/ATP translocase (Aat) from *R. rugosa* and determined its sequence on the X, Y, Z, and W chromosomes of different populations.
Among the differences of the Z and W sequences, the 543rd nucleotide of the W gene (Guanine) corresponding to the 550th nucleotide of the Z gene (Adenine) is useful to distinguish the Z from the W gene by PCR–RFLP. This because a difference provides an *Mbo*I restriction site sequence, GATC, on the W gene which is missing from the corresponding position on the Z gene (AATC). So that, after selective PCR for exon 2 of AAT gene and successive enzymatic digestion with *MboI*, samples can be sexed by examining the electrophoresis profiles: 2 distinct bands for the female (heterogametic sex) and only one in males (homogametic sex).

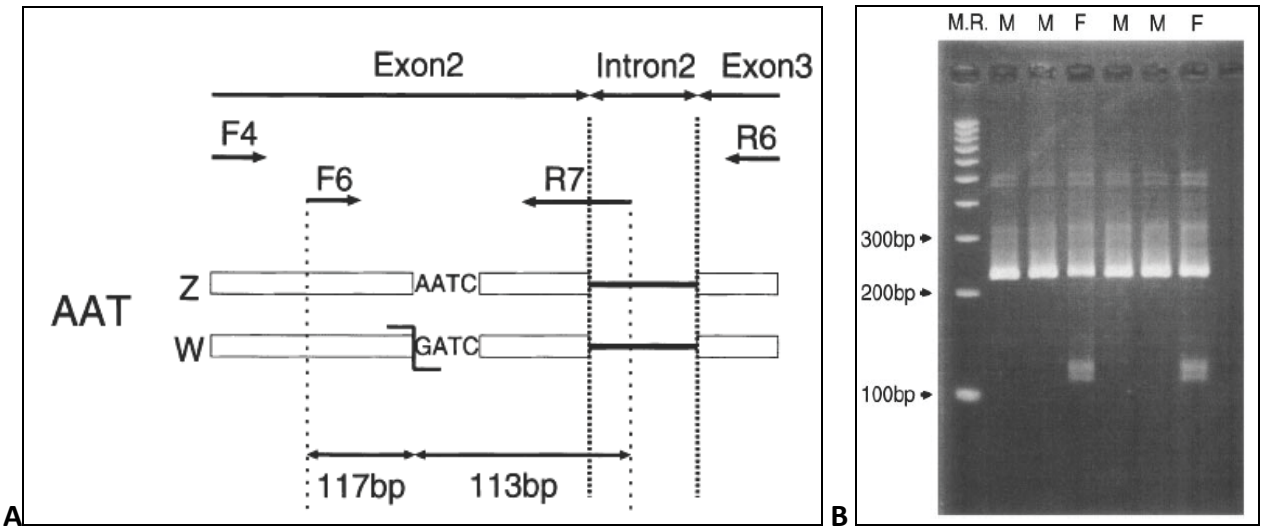

Figure 3.2 : A)scheme showing DNA fragments amplified by nested PCR and digested by restriction enzyme *Mbo*I. F4 and R6 are primers for primary PCR and F6 and R7 are primers for secondary PCR. Amplified fragments from the W gene have a *Mbo*I restriction site, GATC, which is replaced by AATC in the Z gene. B) Agarose gel electrophoresis profiles of DNA fragments amplified by AAT-specific primers and restricted with *Mbo*I. M.R. 100 bp molecular ruler. M: The pattern characteristic of male with no W chromosome. F: The pattern given by female with AAT on the W chromosome (from Sakisaka et al.2000) [147].

To verify the hypothetical role of the AAT as sex-linked gene in *Bufo bufo* we use the same experimental approach.

i) Sequence alignment and primers.

First of all we compared the AAT homologues genes sequences from different amphibian species in order to design forward and reverse primers to amplify the exon 2 in *B.bufo* specimen.
For a comparative analysis of the AAT gene in different species we adopted a multiple sequence alignment. AAT sequence from *R.rugosa* (different populations) *R.sylvatica, X.tropicalis, X.leavis* were aligned utilizing Clustal W software (http://www.ebi.ac.uk/Tools/clustalw2/).
Sequences derived from Genbank: *Rana rugosa* AB008462 "Z1", AB008461 "Y", AB008460 "X", AB008459 "X1", AB008458 "W", AB008457 "Is" , AB008463 „Z2", AB0056 "HI", *Xenpus tropicalis* NM_203582*, Xenopus leavis,* BC043821, *Rana sylvatica* U44832.

CLUSTAL W (1.83) multiple sequence alignment

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AATRrZ2                 ATGACCGACGCAGCAATCTCCTTCGCCAAGGACTTCTTGGCCGGCGGTGTGGCCGCTGCT 60
AATRrZ1                 ATGACCGACGCAGCAATCTCCTTCGCCAAGGACTTCTTGGCCGGCGGTGTGGCCGCTGCT 60
AATRanarugosaX          ATGACCGACGCAGCAATCTCCTTCGCCAAGGACTTCTTGGCCGGCGGTGTGGCCGCTGCT 60
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AATRanasylvatica        ATGACCGACGCAGCTAACTCCTTCGCCAAGGACTTCTTGGCCGGCGGTGTGGCCGCTGCT 60
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xenopuslaevis     F1A   ATGACYGACGCAGCHAWCTCTTTCGCCAAGGACTTCTTGGCCGGCGGTGTGGCCGCAGCT F1
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AATRrY                  GCAAGCAAACAAATCACAGCAGACAAGCAATACAAAGGAATCATGGACTGTGTTGTCCGT 180
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Xenopustropicalis       GCAAGCAAACAGATCACCGCAGACAAGCACTACAAGGGCATCATGGACTGTGTTGTCAGA 180
xenopuslaevis           GCAAGCAAACAGATCACCGCAGACAAGCATTACAAGGGCATCATGGACTGCGTTGTGAGA 180
                        *********** ***** ** ******** ***** ** ** ******** *****  *

                              5'GTTTGCTTGCATGTTGGAC3' R2
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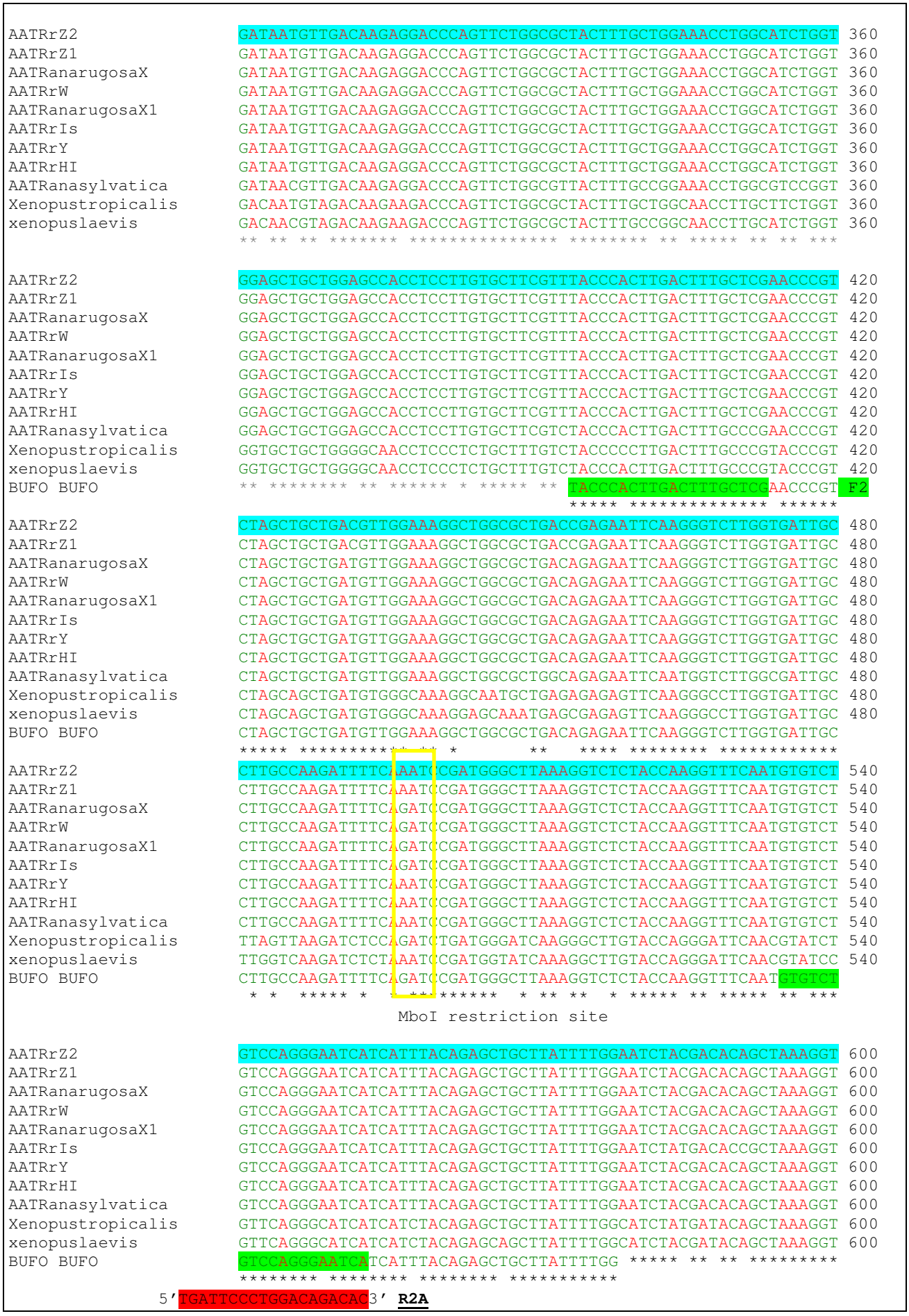
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Xenopustropicalis CTAGCAGCTGATGTGGGCAAAGGCAATGCTGAGAGAGAGTTCAAGGGCCTTGGTGATTGC 480
xenopuslaevis CTAGCAGCTGATGTGGGCAAAGGAGCAAATGAGCGAGAGTTCAAGGGCCTTGGTGATTGC 480
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xenopuslaevis GTTCAGGGCATCATCATCTACAGAGCAGCTTATTTTGGCATCTACGATACAGCTAAAGGT 600
BUFO BUFO GTCCAGGGAATCATCATTTACAGAGCTGCTTATTTTGG
5’TGATTCCCTGGACAGACAC3’ R2A

Table 3.2: sequence alignment.

j) Amplification conditions.

Amplification reactions were performed in 500mM KCl,150mM Tris-HCl (GeneAmp 10X PCR Gold Buffer, Applied Biosystems), 3mM $MgCl_2$, 0.25 mM dNTP Set (Fermentas) , 0.6 µM primer (Sigma Genosys), 2 ng template DNA and 1.5 U Taq polymerase (AmpliTaq Gold, Applied Biosystems) in a final volume of 25 µl. To amplify a partial sequence of the exon 2 (about 200 bp), primers F2→GTCCAACATGCAAGCAAAC and R2→GTTTGCTTGCATGTTGGA (Sigma Genosys) were used.
Primers variables (Len =length, Tm = melting temperature, %GC = percentage in C,G) were calculated using DNA calculator (http://www.sigma-genosys.com/calc/DNACalc.asp) software.
DNA amplification were performed in a thermal cycler programmed as follows: 94°C for 5 minutes followed by 45 cycles consisting of 94°C for 1 min, 56°C for 1 min and 72°C for 2 min.
A final extension was carried out for 7 min. One negative control (absence of template DNA) was performed for each set of amplifications. PCR products were separated by electrophoresis in 1,4% agarose gel in 1X TBE buffer. Molecular sizes of the amplification products were estimated by using a 0,1-10,0 Kb DNA ladder. Gel were stained in ethidium bromide solution and observed under UV light.

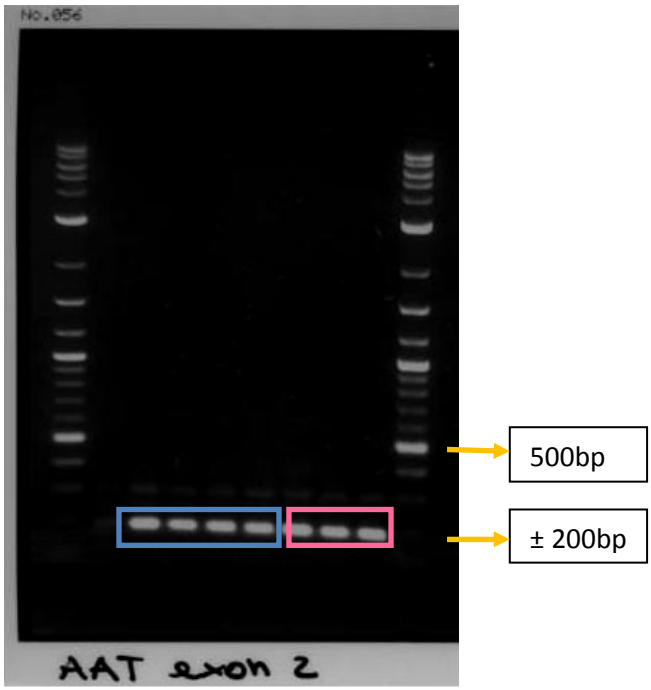


Figure3.3. Amplification of Aat, exon 2. In blue males , pinkfemales.

k) Purification of the PCR products.

PCR products of *Bufo bufo* male and female specimen were extracted and purified in agreement with the Qiagen QIAquick PCR purification kit, and then directly sequenced (Biofab research).

l) Results.

Sequences of AAT gene, exon 2, in male (*Bufo bufo* 1) and female (*Bufo bufo* 5) showing the *MboI* restriction site.

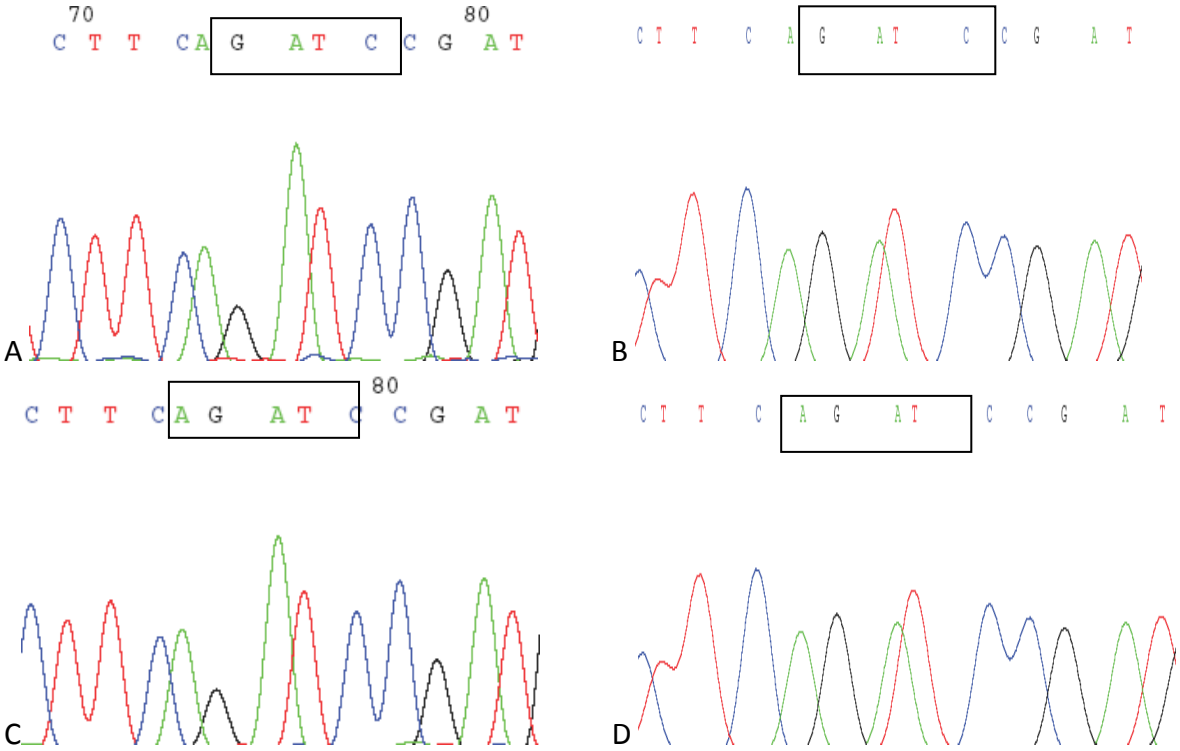


Figure 3.4: partial sequence of AAT gene in *B.bufo*. AAT exon2 gene sequences with primer F2 in *B.bufo* samples 1, male (A)and 5 female(C),and with primer R2 (B male,D female).Black rectangles shows the *MboI* restriction site 5'→3'

The sequence comparison of the AAT (ADP/ATP transolcase) exon 2, from the homogametic sex (ZZ male) and the heterogametic sex (ZW female) don't show any difference in their nucleotides composition as seen in other anuran. No heterozigosis is observed : we can't use this methodology to discriminate the sex of the organisms.

m) Bio-molecular approaches: discussion.

Amphibians employ a genetic mechanism of sex determination according to all available information on sex chromosomes. The inability to distinguish sex chromosomes suggest that a much smaller region may be involved in sex determination in amphibian: for these reasons is essential the research of sex-linked genes.

The different approaches we utilized haven't highlighted any substantial difference between the sexes. Concerning RAPD's primers, probably more selective polymorphisms could be detected modifying the protocol using parental and their offspring genomes. In conclusion, RAPD have some limitations connected to reproducibility, but despite this, it could resolve partially the issues regarding the mechanism of sex determination and the subsequent sexual differentiation.

Concerning the AAT gene, no difference in alleles sequences are identified in the homogametic and heterogametic sex as seen for other amphibian (*Xenopus, R.sylvatica*). For this reason, AAT sequences resulting from male and female specimen are not useful to the purpose of our investigations.

3.4) Sex reversal in amphibians.

a) Introduction.

Another approach we adopted to investigate the alternative pathways in sexual differentiation is to override the genetic program by imposing the development of a unique sexual phenotype. Conditioning the sexual phenotype by external treatments could be a way to obtain information during early stages of development. The most important thing is that the resulting sex-ratio must be unisexual.
For this purpose we tried two different approaches: the first one consist in conditioning the development of a sexual phenotype by hormonal treatments whereas the second and more complex approach is to induce a sexual reverse in *B.bufo* adult males by surgical castration (orchiectomy).
The purposes of the different approaches are quite similar. With hormonal treatments at early developmental stages, the resulting sex ratio could be unisexual as a consequence of an induction exerted by hormones (estradiol or testosterone).
By removing testes with surgical castration, adult males are able to invert their sex resulting in fertile phenotypic females. The breeding test between an "inverted male" with a "normal male" could verify the sexual chromosomal system, adducing experimental evidences.
B.bufo specimens are described as displaying of an ZZ/ZW chromosomal sex determination mechanism, then the expecting offspring resulting from the mating between ZZ "inverted" organisms and ZZ normal organisms should give a 100% ZZ males offspring.

b) Hormonal sex reversal.

Tests of hormonal influence on sex determination have usually involved testosterone and estradiol dissolved in the rearing water. Gallien 1974 [102] has reviewed the results obtained from such treatment, which include orthodox response, paradoxical response (contrary to expectation) and a few refractory species. In general, ranid and hylid tadpoles are masculinized by testosterone and by high doses of estradiol, which at low doses causes slight or temporary feminization. Most other anurans as *Xenopus* and all urodeles are feminized to some extent by estradiol but are not masculinized by testosterone which causes paradoxical feminization in some species but not others. Testosterone usually represses the development of gonads in this group, sometimes to such an extent that paradoxical feminization occurs by the recovery of an ovary after the end of the treatment (Wallace et al.1991) [103]. One common side effect of these steroid hormonal treatments is a suppression of oviduct growth, resulting in sterility of feminized specimens. Consequently, there are only a handful of cases where breeding tests have demonstrated the success of this experimental manipulation resulting in the attribution of which sex is heterogametic.
The variety of hormonal effects might be interpreted by assuming that amphibians are capable of converting excessive amounts of an administered hormone into another one more suitable for

their needs. The conversion of testosterone to estradiol by aromatase has been reported in a wide variety of vertebrates, and in principle it should be a reversible reaction.
Aromatase activity has been related to sex determination and masculinization at high temperatures in *Pleurodeles waltl* (Chardard D., Desvages G., Pieau C. and Dournon C. 1995) [104]. Steroid hormones are not considered the basic mechanism of sex determination in amphibians they are necessary components of the differentiations pathways, as they can reinforce or reverse the initial determination, in addition to coordinating secondary sexual characteristics to the primary sex of the gonads.

Genus	System	Estrogen	Androgen
Urodeles			
Ambystoma	ZZ/ZW	+	fem.
Hynobius	?	+	− or fem.
Pleurodeles	ZZ/ZW	+	fem.
Triturus	XX/XY	+	−
Anurans			
Buergeria	ZZ/ZW	masc.	+
Bombina	XX/XY	−	−
Hyla	XX/XY	+	−
Pelobates	?	+	−
Pseudacris	?	+	+
Rana	XX/XY	+ or −	+
Xenopus	ZZ/ZW	+	−

Table3.3: Summary hormonal sex reversal. The response are classified as effective orthodox (+), minor or none (-) or paradoxical (masc. or fem.) (from Wallace,1999)

c) Hormonal treatments in B.bufo tadpoles at early developmental stages (III1...III9).

Fertilized eggs derived from a single couple of *B.bufo* specimen were allowed to grow to tadpoles for use in experiments. Tadpoles at early developmental stages were reared in dechlorined water and divided into different containers. A total of 20 tadpoles were reared in each container in order to estimate the effects of hormonal treatments on the sex ratio and on the mortality. Control samples were reared in the same conditions (water temperature, water volume, alimentation).
Synthetics hormones (testosterone and estradiol) were dissolved in 100% EtOH (100mg T/E in 100 cc EtOH) to produce a 1mg/ml hydro-alcoholic solution. According to the developmental stages (III1, III4, III7, III9), hormonal treatments were performing for 24 hours by utilizing an 0.05% hydro-alcoholic solution, with a final hormonal concentration of 20 µg/l.
At the complement of metamorphosis (stage IV17), tadpoles were fixed in Bouin's fluid and embedded in paraffin. Serial cross section (7µm) were made by using a microtome (Jung AG) and then stained with hematoxilyin and eosin. The determination of sexes was identified by optical microscopic analysis.

d) Results and discussion.

	N	N_0	M	F	sex-ratio (M:F)		*X2 sex ratio*	P	%S
T	80	23	11	12	0,9	1	0,04	NS	29
E	80	28	6	22	0,3	1	9,14	P<0,01	35
C	80	53	29	24	1,2	1	0,47	NS	66

Table 3.4: **hormonal treatments experimental results**. T=testosterone, E=estradiol, C=control, n=number of specimen reaching the complement of metamorphosis, M,F males or females, X^2 chi quadrate, %S = survival express as percentage.

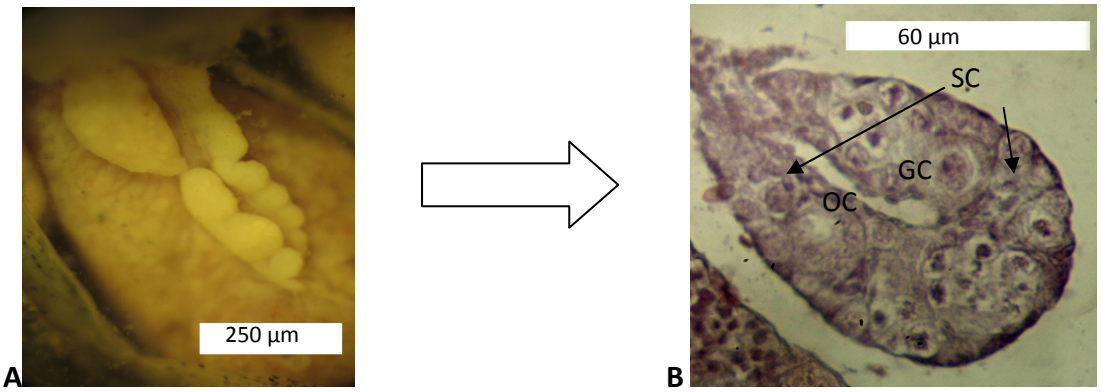


Figure 3.5: female gonad. Pictures showing a female gonad fixed in Bouin's fluid (A) and stained with hematoxilyin and eosin (B). OC = ovarian cavity, GC = germinal cell, SC = somatic cell.

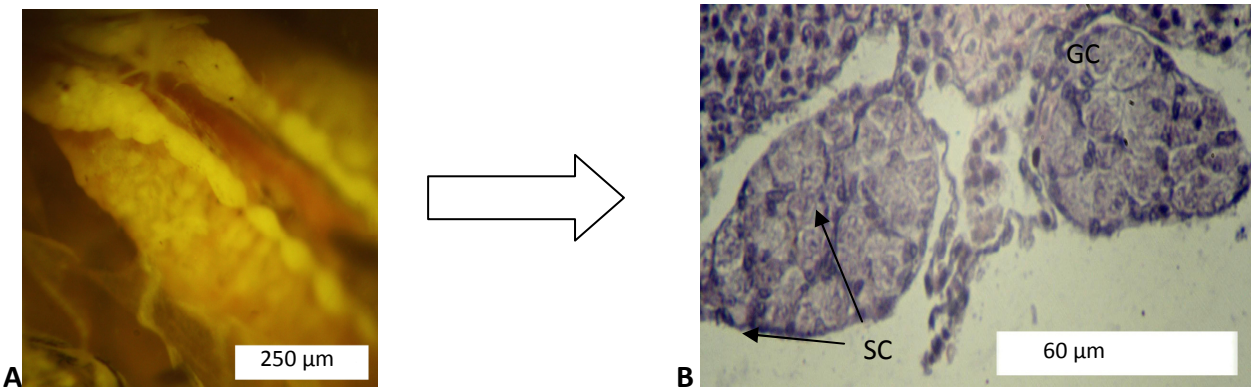


Figure 3.6: male gonad. Pictures showing a male gonad fixed in Bouin's fluid (A) and stained with hematoxilyin and eosin (B). GC = germinal cell, SC = somatic cell.

The effects of hormonal treatments with estradiol and testosterone can be summarized as follow:

- the mortality rate seems to be the limiting variable; specimens treated with hormones shows a higher mortality if compared to control (table 3.4): hormonal treatments seems to exert a toxic action;
- by imposing a sex-ratio of 1:1 to calculate X^2 distribution, the observed sex-ratio in specimen treated with estradiol is statistically significant ($p<0,01$). Estradiol seems to act by stimulating the development of female phenotypes;
- to verify as the toxic action is exerted, we impose a sex ratio of 1:1 and the mortality rate equal to control samples to calculate X^2 distribution:

a) X^2 distributions in males treated with estradiol or with testosterone are not statistically significant: we can argue that both hormonal treatments are toxic in male specimen;

b) X^2 distributions in female treated with testosterone is not statistically significant: we can argue that with testosterone treatments are toxic in female specimen;

c)X^2 distributions in female treated with estradiol is statistically significant ($p<0,01$):we can argue that with estradiol treatments are not toxic in female specimen.

While hormonal treatments with testosterone seems to be toxic for male and female specimen, estradiol seems to act in a differential manner resulting toxic only on males specimen; during their embryonic life, genotypic female tadpoles survive whereas genotypic male tadpoles die before reaching the stage IV17.

However, hormonal treatments seems not induce a unisexual phenotype, and this poses serious limitations for the purposes of research. A hypothesis to test is to anticipate or prolong the exposure at estradiol increasing the number of organisms, or to rear tadpoles in presence of estradiol at low temperatures (because low temperatures seem to act as epigenetic factor inducing a feminizing effect on the larvae in development).

e) Sex-reversal by surgical castration.

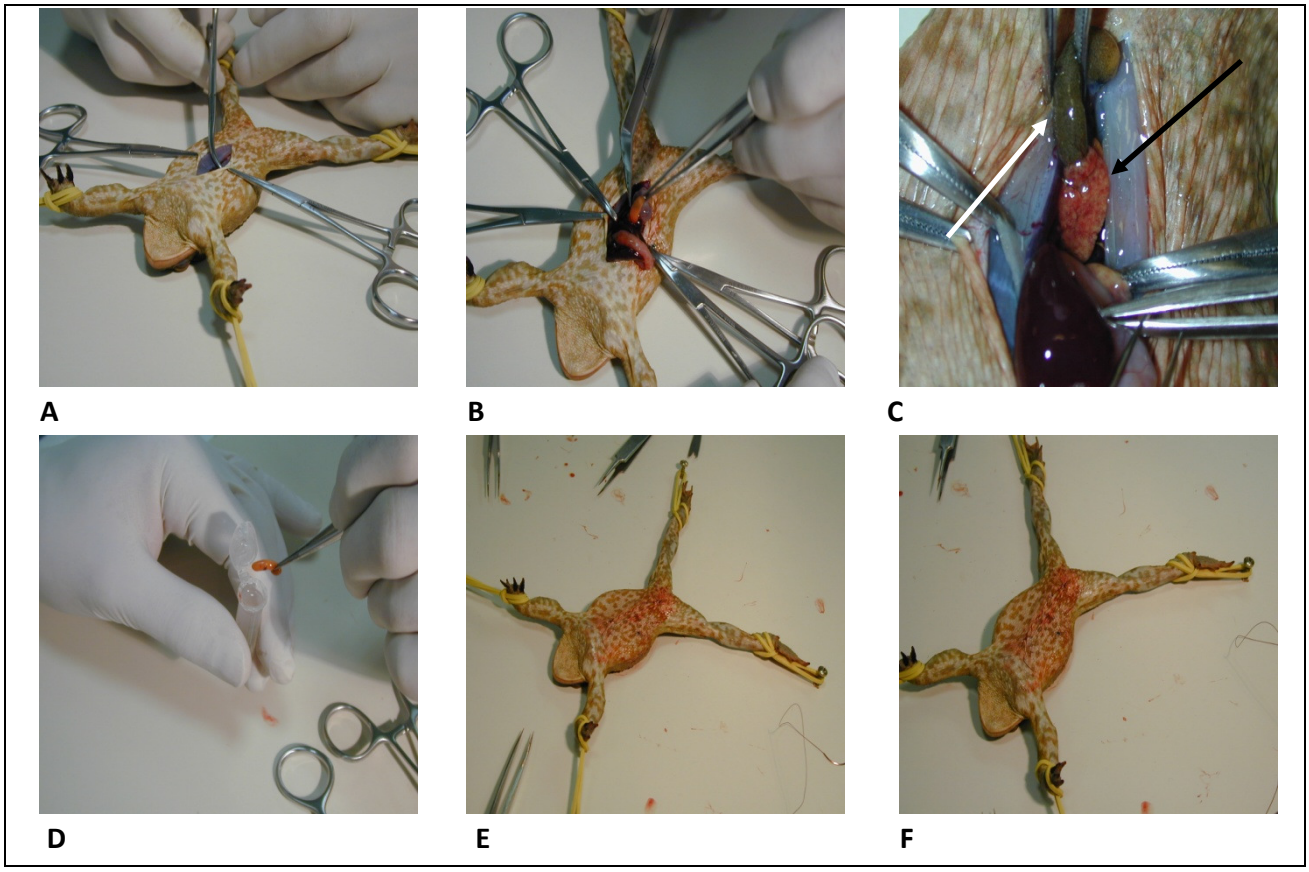
Bufonids toads resemble undifferentiated races of *Rana* in that all tadpoles develop ovaries which become compressed into Bidder's organ in front to definitive gonads (ovary or testis) at metamorphosis. Ponse (1941) [105] found that castrated adult *Bufo vulgaris* of either sex can convert Bidder's organ into a functional ovary and thus become a phenotypic fertile female. In the case of castrated males, which presumably change sex in the absence of the normal androgen, repeated mating of inverted males with normal males over many years allowed Ponse to accumulate about 1000 offspring which were all males. Based on her experimental evidences Ponse concluded that *Bufo* specimens display a ZZ/ZW genetic system in which males represented the homogametic sex and female the heterogametic sex. Based on similar approaches Harms (1926) [106] obtained a different result: by mating normal and castrated males, he accumulate a bisexual offspring. From these experimental evidence Harms argued that bufonids display an XX/XY sex determining system. The breeding test with sex-reversal male toads have not completely elucidated the chromosomal sex determining mechanism in bufonids; for these reasons data must be confirmed because of differences in chromosomes sex determining mechanism can occur in different populations as reviewed for *R.rugosa* (Miura et al.1998)[101].

Many parallels are known to exist between ovarian and bidderian oogenesis. Zaccanti and Gardenghi (1968) [110] found that, in adult *B.bufo*, an identical three-yearly period is spent from the beginning of meiosis to the egg maturation in ovary, or to the oocyte degeneration in Bidder's organ. Utilizing the terminology suggested by Colombo (1953) [111] and Vannini (1954) [112] they specified that both ovarian and bidderian oogenesis begin when reserve oogonia become very young oocytes initiating the meiosis, at the "first growth period" a process which occurs continuously throughout the first year of the oogenetic cycle. The stages of the "second growth period" begin during a part of the first year, are protracted during the second year and are concluded at the beginning of the third year. During the third year the ovarian oocyte begin

vitellogenesis so reaching the “third growth period” while in the meantime the male bidderian oocyte begin to degenerate. Ovary differ from the male Bidder’s organ mainly in the vitellogenetic phase, present in the ovary and absent in the Bidder’s organ. As reported by others, estrogens induce synthesis of vitelline proteins in the liver of amphibians, just as in the majority of the other vertebrates. Thus it is feasible that in *B. bufo* adult males the production of androgens by the testes somehow block vitellogenesis. However this blocking power of the androgenic hormones might be overcome by experimental treatments with estrogens. In fact, in normal adult male specimens of *B.bufo* Zaccanti and Gardenghi (1970) [113] succeeded in inducing the usually abortive bidderian oocytes to reach vitellogenesis phase as a result of prolonged administration of the synthetic estrogen diethylstilbestrol dipropionate (DES), whose effect is quite similar to that of estradiol in mammals, birds and teleosts. Zaccanti and Tognato (1976) [114] have reviewed that different concentration of DES in orchiectomized males have different effects. Using a dosage of 1ug DES/week (as in our experiments) after a period of 9-10 months from the orchiectomy and treatments, the macroscopic examination of the Bidder’s organ shows a greater increase in volume, and a great number of the usually abortive large diplotenic oocyte initiate a massive vitellogenesis reaching the “third growth period”.

f) Surgical castration of B.bufo adult males.

Adult frogs males taken from our laboratories were previously anesthetized using chloroform, rinse with water and then placed in the dissection pan. The frog should be lying on its dorsal (back) side with the belly facing up by securing each of the four limbs to the pan with elastics. Once the legs of the frog were securely blocked, cut the skin. The way to start cutting a frog is to cut at the bottom of the abdomen and then make along the center, or midline of the frog, a vertical cut bisecting it equally (A). Once finished the incisions between the front and rear legs of the frog separate the skin flaps from the muscle below. To do this pick up the flap of skin with needle-nose pliers, and use a sterile scalpel to help separate the skin from the muscle below. Now that the skin has been cut, begin the abdominal muscle incision by using the forceps to lift the muscle midway between the rear legs of the frog. Using the scissors, carefully continue the incision near up the midline of the frog, but do not cut too deeply as to damage the organs. Separate muscle and organs by opening up the frog's body cavity therefore exposing the abdominal region (B). Reach carefully the testis by moving bowels (C) and then remove the them very by using a scalpel or forceps (D) paying attention to not sever some vessel. Suture up the abdominal cavity using sterile and re-absorbable wire (E) and finally suture up the skin by using not re-absorbable wire (F).



Figures 3.7: steps during orchiectomy. Principal steps in surgical castration of *B.bufo* adult males. In picture C black arrow indicates Bidder's organ, white arrows indicates the testicle.

g) Hormonal treatments (DiEthylStilbestrol dipropionate).

After surgical castration, samples of *B.bufo* were divided into different groups: in the first one, composed by orchiectomized males weekly injected with a solution containing 1ug/ml DES (diethylstilbestrol dipropionate, Flucka). DES is a synthetic estrogen whose effects is similar to that of estradiol in mammals, accelerating the development of bidderian oocytes to reach the vitellogenetic stage.

The second group consisted in orchiectomized but not injected males to verify the different Bidder's development and to value if the injection could represent an additional stress. According to Harms (1921,1926) [106,107] and Ponse (1925,1927) [108,109], in orchiectomized males not subjected to estrogenic treatments, the oogenetic process should spontaneously occur very slowly: from three to seven years after orchiectomy.

Finally we used also normal males as control samples. A total of 15 elements were utilized in this experiment; the code number of each organism derives from a chip injected in lymphatic pouch.

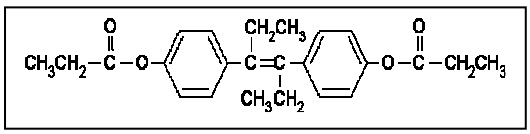
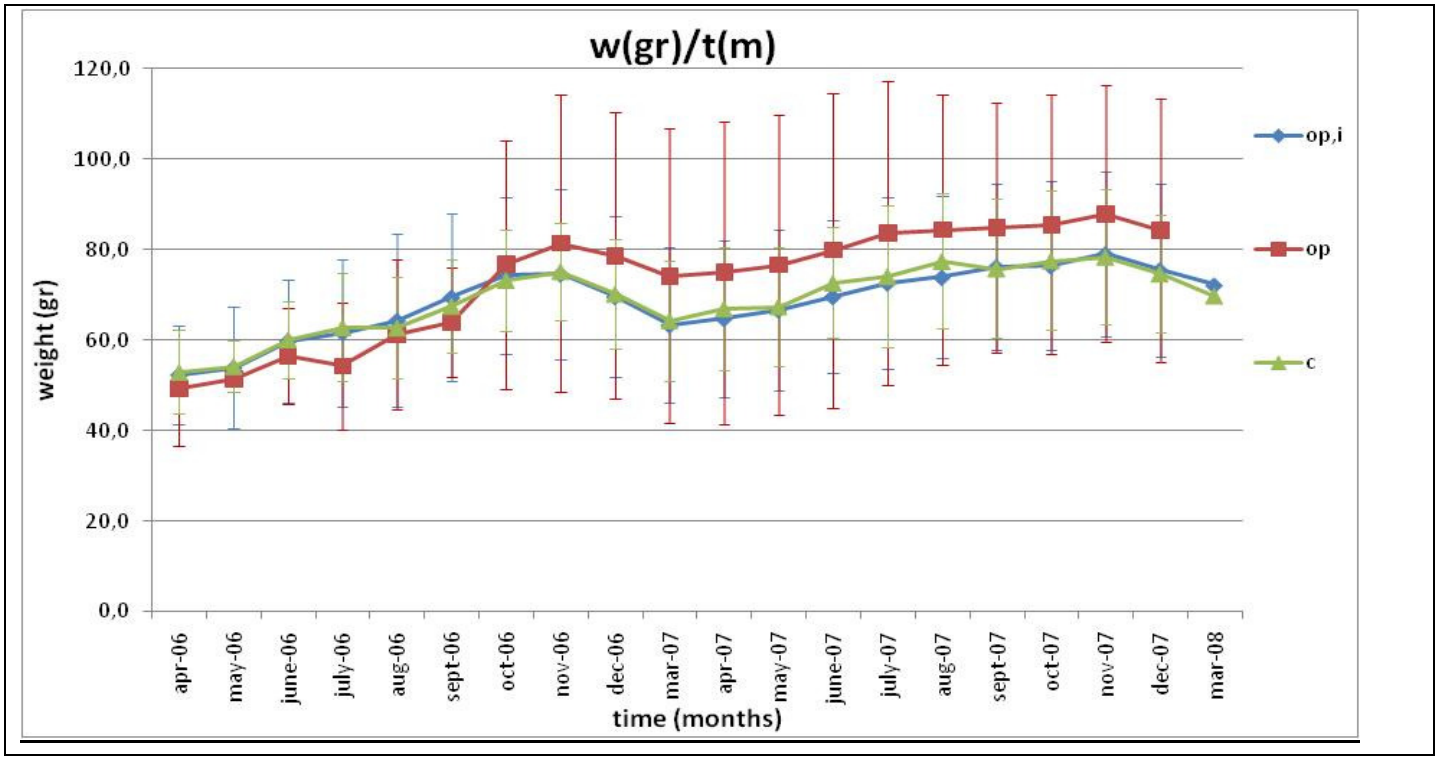


Figure 3.8: DiEthylStilbestrol dipropionate

code number	orchiectomized (op)	DES (i)
8545FC (op,i)	x	X
826182 (op,i)	x	X
406CFC (op,i)	x	X
F35282 (op,i)	x	X
D7C3BC (op,i)	x	X
32A2FC (op,i)	x	X
280682 (op,i)	x	X
6D55FC (op)	x	
7OOE82 (op)	x	
D1A2FC (op)	x	
E00982 (op)	x	
7DDCFC ©		
982CFC ©		
F353FC ©		
16D182 ©		

Table 3.9: specimens. Code numbers are referred to each sample. op=orchiectomized, op,i=orchiectomized+DES, c=control

h) Results and discussion.



Graph 3.1: increase in weight as a function of time. Each point represent the average of the different clusters. Blue line= operated and injected samples, red line= operated samples, green line= control samples.

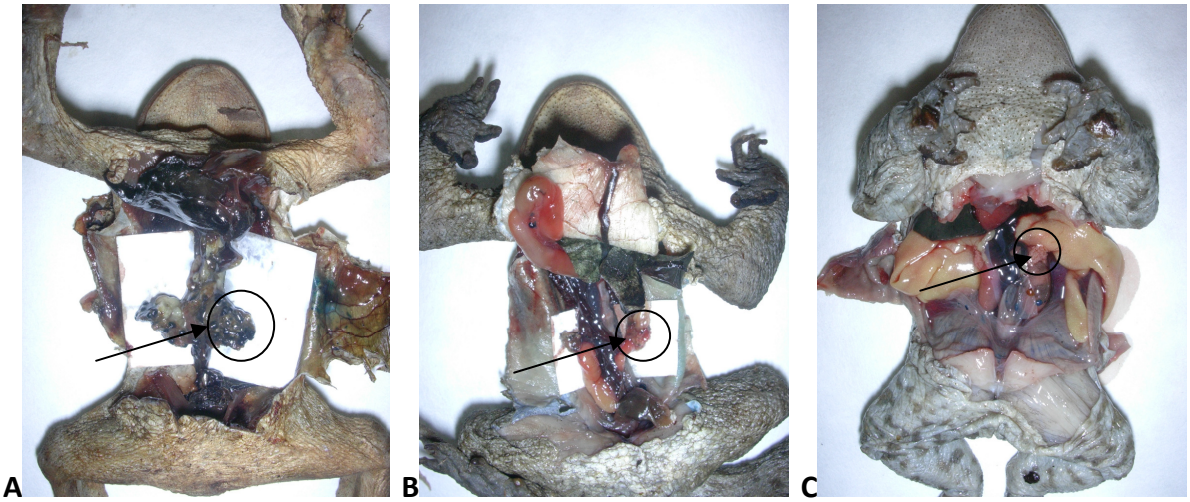


Figure 3.10: specimens after orchiectomy (A,B) and control sample(B). Figure A is referred to an orchiectomized and injected sample, B to an injected sample. C, control male.

After surgical castration, males were reared for almost 2 years with the purpose to obtain an unisexual offspring by mating "inverted" and "normal" males.

Graph 1 shows a common trend in size increasing, during these years the animals had not shows particular problem if compared to the control samples. To value the stress induced by castration we observed the eating behavior: it was very limited in time, from few days to a week then castrated males respond in equal manner to controls.

After winter hibernation, both castrated and normal males presented sexual secondary characters (vocalize and thumb's call) and did not mating. Only two samples achieved the second spring (one control and an inverted); also in this case the inverted male presented the thumb's call and did not mate. Both specimen died late in march.

By dissecting the specimen, we can confirm the results obtained by Zaccanti and Tognato (1976) [114]. Using a dosage of 1ug DES/week the macroscopic examination of the Bidder's organ two years after orchietomy show a greater increase in volume(fig 3.10 A), and a great number of the usually abortive large diplotenic oocyte initiate a massive vitellogenesis reaching the "third growth period". In orchiectomized males, the Bidder's organ appear much greater in volume than those of intact control males but oocytes don't reach the vitellogenetic phase (fig 3.10 B).

Inverted *B.bufo* specimen were not able to reach the mating, for this reason it was impossible to verify the heterogametic sex in *B.bufo* by breeding tests.

The causes of death are unknown. Treatments with hormones can reduce the normal life by producing tumors in various districts (with a dose dependent trend), but the comparison between inverted and normal males showed a quite similar rate of body growth and a longevity.

Probably the limiting condition is related to the captivity.

i) Sex reversal in amphibians: discussion.

Searching missing pieces in developmental mechanism by using sexual reversal have not produce the expected results.

Hormonal treatments have underline different evidence. The mortality rate seems to be the limiting variable; specimens treated with hormones shows a higher mortality if compared to control (table 3.4): hormonal treatments seems to exert a toxic action. Comparing the resulting sex ratio in specimen treated with estradiol, data underline a statistically significant difference trough a female development. Moreover, while hormonal treatments with testosterone seems to be toxic for male and female specimen, estradiol seems to act in a differential manner resulting toxic only on males specimen; during their embryonic life, genotypic female tadpoles survive whereas genotypic male tadpoles die before reaching the stage IV17. Exogenous dosages of estradiol could alter the male developmental pathway increasing the mortality rate in male specimen. However, hormonal treatments seems not induce a unique sexual phenotype, and this poses serious limitations for the purposes of research. A hypothesis to test is to anticipate or prolong the exposure at estradiol increasing the number of organisms, or to rear tadpoles in presence of estradiol at low temperatures (because low temperatures seem to act as epigenetic factor inducing a feminizing effect on the larvae in development).

Concerning sexual reversion by castration, the incapability to produce an offspring by the mating between “normal” and “inverted” males have not give us information about the chromosomal sex determining mechanism. For this reason it was not possible to investigate the gonadal development of a unisexual offspring, and so it was not possible to study the interaction between somatic and germinal districts during male development. We can confirm that orchiectomy induce a sexual reversion and that organism treated with DES accelerate the bidderian oogenesis .

Using a dosage of 1ug DES/week the macroscopic examination of the Bidder’s organ two years after orchietomy show a greater increase in volume, and a great number of the usually abortive large diplotenic oocyte initiate a massive vitellogenesis reaching the “third growth period”. In orchiectomized males, the Bidder’s organ appear much greater in volume than those of intact control males but oocytes don’t reach the vitellogenetic phase.

3.5) Sexual differentiation: methodological approaches.

a) Introduction

In bisexual animals sexual differentiation represent the process of development of differences between males and females of the same species starting from an undifferentiated zygote and resulting in sexual dimorphism. Alternative phenotypes may be induced by specific genes, reflecting their differential expression in the gonadal primordium, which produce different hormonal effects culminating in anatomical differences. In anuran, sexual dimorphism is termed reverse sexual dimorphism because of a bigger size of female specimen respect males organisms.
To investigate the differences in the alternative developmental pathways, we used three different approaches.
First of all, we have conducted a study in confocal laser scanning microscopy in order to define the alternative architectures in male and female gonads, highlighting the cytoskeletal organization. The purpose of this investigation is to describe the differential morphology of gonadal compartments by visualizing the distribution of α-tubulin in males and females specimen at the complement of metamorphosis (stage IV17).To investigate more specifically the spatial distribution of the germinal cells and their interaction with the soma in the alternative gonadal phenotypes, we utilized the same technique by detecting the spatial distribution and the specific localization of *Vasa* protein.
Finally, we have conducted a study in whole mouth in situ ibridization (wmISH) in order to understand the differential expression of *Sox9* during the alternative gonadal development. The purpose of this approach is to understand if *Sox9* can be used as a marker in the early development of male phenotype and to recognize if the role exerted by *Sox9* in mammals can be considered as a conservative mechanism also in amphibians.

b) Immuno-detection and localization of α- tubulin in *B.bufo* specimen (stage IV17) by confocal laser scanning microscopy.

Confocal laser scanning microscopy (CLSM or LSCM) is a technique for obtaining high-resolution optical images. The key feature of confocal microscopy is its ability to produce in-focus images of thick specimens, a process known as optical sectioning. Images are acquired point-by-point and reconstructed with a computer software, allowing three-dimensional reconstructions of topologically-complex objects. In our experiment we have detect the distribution of α-tubulin in the cytoskeleton and Vasa protein as marker of germ cells.

c) Cytoskeleton: generalities.

The cytoskeleton is typical to eukaryotic cells. It is a dynamic three-dimensional structure that fills the cytoplasm determining the morphological organization of the cells. The cytoskeleton is now no longer considered to be a rigid scaffold, but instead is viewed as a complex and dynamic network of protein filaments that can be modulated by internal and external cues as response to the cell cycle and enviromental stimulation: it acts as both muscle and skeleton, for movement and stability.

The long fibers of the cytoskeleton are polymers of subunits that interact themselves and with plasmatic and nuclear membranes. The functional roles of these structures are manifold and do not only sustain the cell's shape and its mechanical scaffold. They are involved in several basic cellular processes such as segregation of genetic material during cytokines and karyokinesis, intracellular transport, positioning of cell organelles, extracellular transport by means of cilia, and movement of cells by means of flagella. They are also implicated in the correct localization of proteins for the specification of the germ cells.

The primary types of fibers comprising the cytoskeleton are three different structural proteins that differ both in diameter and composition called microfilaments, microtubules and intermediate filaments.

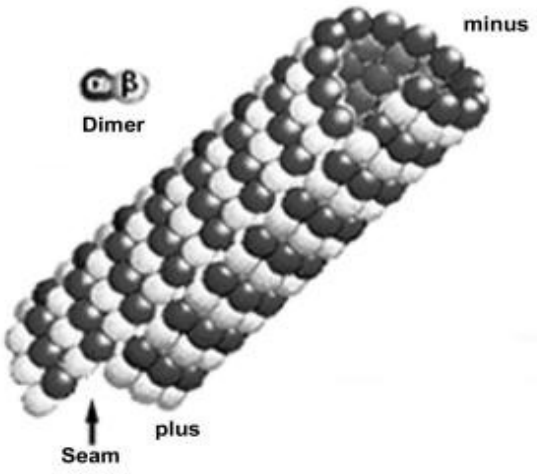
Microfilaments are variable in length and their diameter is about 7-9 nm. These filaments are cross-linked into networks or bundles. In most cases a shell of microfilaments are commonly found in the cell cortex, supporting the plasma membrane. They are composed predominantly of a contractile protein called actin plus attached proteins like cross-linkers.

Actin is a globular 42-kDA protein (G-actin) found in all eukaryotic cells present as 15-20% of the total cellular proteins. In presence of metallic ions (MG^{2+}, K^{+}) G-actin subunits assemble into long filamentous polymers called F-actin.

Microfilament's association with the protein myosin is responsible for muscle contraction. Microfilaments can also carry out cellular movements including gliding, contraction, and cytokinesis.

Intermediate filaments (IF)are about 10 nm diameter and provide tensile strength for the cell. Some IF are homopolymeres of one protein, some are heteropolymeres of two or more proteins. All intermediate filament proteins (67 human genes known) have a common basic structure with main differences at both ends. They form homogenous, apolar fibers with diameters of 10-12 nm.

Some IF proteins are ubiquitous (vimentin) others are restricted to neurons of the central nervous system (neurofilament proteins), muscle (desmin, syncoilin) or epithelial cells (keratin).
As well as the expression the organization of the intermediate filaments is cell-type-dependent.
In many epithelial cells filaments are distributed all over the cytoplasm and attach to the nucleus, while in primary fibroblasts vimentin is orientated towards the periphery and spans neither the whole cytoplasm nor is it connected to cell-cell-adhesion sites.
The intermediate filaments network is dynamically influenced by a bunch of other proteins. The IFs provide mechanical stability as well as they take part in the assembly of the nuclear envelope or neurofilaments contribute to the radial growth of neurons.
Microtubules act as a scaffold to determine cell shape, and provide a set of "tracks" for proteins, cell organelles and vesicles to move on. Microtubules also form the spindle fibers for separating chromosomes during mitosis. When arranged in geometric patterns inside flagella and cilia, they are used for locomotion.
Microtubules are intracellular dynamic polymers made up of evolutionarily conserved polymorphic alpha/beta-tubulin heterodimers (Amos and Klug 1974) [115] of 55 k-DA, and a large number of microtubule-associated proteins (MAPs). One alpha- and one beta-tubulin form a heterodimer. Long chains of these heterodimers compose protofilaments, wherein always a alpha-tubulin is followed by a beta-tubulin.
The microtubules consist of 13 protofilaments associate site by site to form a tube and have an outer diameter 25 nm. Microtubules have their intrinsic polarity; highly dynamic plus ends and less dynamic minus ends. Because of the dimeric character of tubulin, one end of the protofilament is terminated by an alpha subunit and at the opposite one by a beta subunit. This provides the protofilament a certain kind of polarity. Within the microtubule, the protofilaments are associated laterally with same polarity. Therefore, the microtubule also appears as a polar structure with a plus and a minus end. Polarity is a very important feature for microtubule functioning. It is the basic property for direction-dependent cellular events, as vesicle transport.



Microtubules assemble and disassemble dependent on the temperature and the surrounding tubulin concentration and is also regulated by microtubule-associated proteins (MAPs). Microtubule polymerization is reversible and a populations of microtubules in cells are on their minus ends either growing or shortening, this phenomenon is called dynamic instability of microtubules. On a practical level, microtubules can easily be stabilized by the addition of anti-cancer drugs such as Taxol at room temperature for many hours.
The arrangement of microtubules in cells is determined by microtubule-organizing centers (MTOCs). These MTOCs consist of different proteins like gamma-tubulin and pericentrin. The microtubules direct their (-) end to the MTOC. In general every eukaryotic cell has a primary MTOC, the centrosome.

Microtubules are required for vital processes in eukaryotic cells including mitosis, meiosis, maintenance of cell shape and intracellular transport. Microtubules are also necessary for movement of cells by means of flagella and cilia.
An important role of microtubules is providing a pathway for intracellular movements of organelles and proteins. This is done by motor proteins (kinesins and dyneins) under consumption of adenosine triphosphate (ATP). Most kinesins carry their cargo along microtubules in (+) direction, while dyneins do so in (-) direction.
The **alpha-tubulin** (relative molecular weight around 50 kDa) is globular protein that exists in cells as part of alpha/beta-tubulin dimer or it is polymerized into microtubules. In different species it is coded by multiple tubulin genes that form tubulin classes (in human 6 genes). Expressed tubulin genes are named tubulin isotypes. Some of the tubulin isotypes are expressed ubiquitously, while some have more restricted tissue expression. Alpha-tubulin is also subject of numerous post-translational modifications. Tubulin isotypes and their posttranslational modifications are responsible for multiple tubulin charge variants - tubulin isoforms. Heterogeneity of alpha-tubulin is concentrated in C-terminal structural domain.

Taking these functions into account it is plausible that the disruption of the cytoskeleton or even subtle changes of its integrity may cause pathological outcomes.

d) Cytoskeleton organization in Xenopus oocytes.

Several lines of evidence suggest that oocyte MTs take part in developmental function including establishing and maintaining of the animal-vegetal polarity of oocytes during stages IV-VI of oogenesis, transport of developmentally important maternal RNAs, positioning of the germinal vesicle in the animal hemisphere. MTs also play a critical role in establishment of the dorsal-ventral axis of the developing embryo (Gard et al,1995) [116].

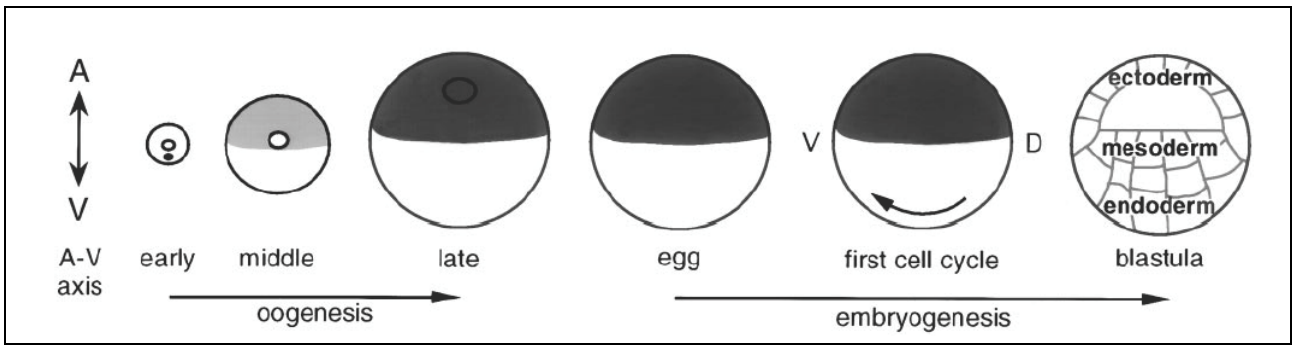


Figure 3.11:a**xis specification during oogenesis and embryogenesis in Xenopus**. A=animal pole, V=vegetal pole. Oogenesis (on the left) is represented as early (stages I-II), middle (stages III-IV) and late (stages V-VI). White circle represent the GV (oocyte nucleus), black circle represent MT (mitochondrial cloud). Embryogenesis (on the right) : a black arrow shows the direction of the vegetal yolk mass during cortical rotation, and the relationship to the future D-V axis (from Monwry,1999) [153].

Differences in developmental potential along the A-V axis have long been viewed as arising from cytoplasmatic localization of maternal determinants. Thus patterning in the embryo is dependent on the prelocalization of mRNAs and proteins within the oocyte. Within the context, specific localized mRNA molecules have been implicated in primary germ layer specification and localized RNAs have been identified as components of the germ plasm as well. Localization of germ plasm, which specifies the germline is a very early indicator of oocyte polarity. It is apparent that the source of germ plasm is the mitochondrial cloud (MC), which is an accumulation of granular-fibrillar material and mitochondria on the presumed vegetal side of germinal vesicle (GV) in stage I oocytes. After the MC breaks down during late stage I early stage II of oogenesis mitochondria and germinal granules are found in vegetal-cortical islands of germ plasm (Mowry and Cote ,1999) [153]. Dynamic reorganizations of cytoskeletal elements also occur throughout oogenesis. These reorganizations can significantly influence the distribution of many organelles and molecules that are transported, anchored, segregated, or otherwise distributed along these elements, and may contribute to regional differences within the cortex and cytoplasm of both the animal and the vegetal hemispheres. In the egg, germ plasm persists as vegetal-cortical islands of yolk-free cytoplasm containing germinal granules associated with large aggregations of mitochondria. During early cleavage, the islands of germ plasm coalesce into larger aggregates, which are eventually inherited by a subset of vegetal blastomeres, the primordial germ cells. During the oocyte development cytoskeletrical components are changing.

Dumont (1972) [117] classified the long diplotene phase of oogenesis in *Xenopus* into six stages, according to the external appearance and cytoplasmic organization of the oocytes. These stages have been commonly accepted as a normal table for oocyte differentiation. However, it should be remembered that oogenesis is not a discrete series of stages, but a continuum of oocyte growth and differentiation; the range of diameters associated with a given stage can vary between individual frogs.

Gard introduced an additional stage termed stage 0 in *Xenopus* oogenesis to distinguish the changes in nuclear, cytoplasmic, and cytoskeletal organization during the transition from pachitene to diplotene.

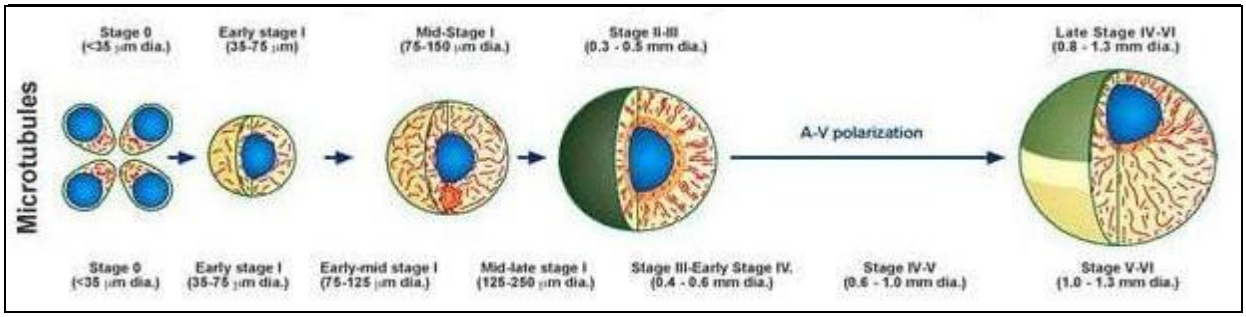


Figure 3.12:summary of MTs organization during the differentiation of *Xenopus* oocytes. During stage 0 oocytes contain a dense network of MTs. By early stage I (35-75 um) the MTs extended throughout the cytoplasm (from Grad,1999)

Primordial germ cells: are diploid germ cell precursors that transiently exist in the embryonic life before they enter into close association with the somatic cells of the gonad and become irreversibly committed as germ cells. PGCs are the descendants of cells that inherited the "germ plasm" found at the vegetal pole of stage VI oocytes. During their migration through the endoderm, hindgut and dorsal mesentery and at the achievement of the genital ridges, PGCs proliferate and can be identified by their round morphology, approximately 15-20 um in diameter, and their lobed nucleus with 1-2 nucleoli.

Oogonia (15-20 um) and stage 0 oocytes (< 35 um): In the ovary of female frogs, PGCs give rise to a self-renewing stem cell population of primary oogonia, from which are derived a population of secondary oogonia committed to embark on the pathway of oocyte differentiation. Secondary oogonia undergo a final series of four synchronous mitotic divisions, resulting in a cluster or "nest" of sixteen oocytes. During interphase, secondary oogonia are similar in appearance to PGCs: they are round, 15-20 µm in diameter, with a lobed nucleus.

Completion of the mitotic divisions of secondary oogonia gives rise to a nest of post-mitotic oocytes in the pre-diplotene stages (stage 0) of the prolonged meiotic prophase I (Coggins,1973)[144]. These early oocytes exhibit a distinct polarity resulting from their final mitotic division. Confocal immunofluorescence microscopy revealed that stage 0 oocytes contain a dense array of MTs concentrated in the cap of cytoplasm located at the narrow end of these pear-shaped cells with the large, round nucleus at the broader, distal end of the cell. Cytoplasmic organelles, including mitochondria, are concentrated in the proximal extremity of the cell. In the nucleus of stage 0 oocyte undergoing meiotic recombination, with the synapsed chromosomes condensing into the classic "bouquet" organization, where synaptonemal complexes are associated with the nuclear envelope on the side of the nucleus facing the cap of cytoplasmic organelles indicating that also the nucleus of satge 0 oocyte is structurally polarized (Coggins,1973)[144]. By late pachytene stage, individual oocytes are becoming surrounded by follicle cells, disrupting the organization of oocyte nests. Following completion of recombination during the pachytene stage of prophase, oocytes enter a prolonged diplotene phase of growth and differentiation and establishment of the A-V axis (Gard et al., 1995) [116].

Stage I (35-300 µm): during the long diplotenic phase, stage I referred to pre-vitellogenic oocyte. Early stage I (35-75 µm diameter) oocyte lost the distinctive polarity of stage 0 oocytes, and appear symmetrical in shape and in cytoplasmic organization with dispersal MTs throughout the cytoplasm.The growing nucleus, or germinal vesicle (GV), moves to the center of the oocyte and cytoplasmic organelles, such as mitochondria, become dispersed throughout the cytoplasm.

Mitochondria begin to aggregate into perinuclear clumps in association with MTs, and by mid-stage I (75-150 µm), oocytes contain one or two prominent mitochondrial aggregates, variously referred to as the Balbiani bodies, mitochondrial clouds (MC). The granular fibrillar germ plasm is associated with the mitochondrial mass of stage I oocytes, as are several maternal RNAs (Gard,1999)[145].

Stage II (300-400 µm): The onset of vitellogenesis, or the production of yolk, marks the beginning of stage II of oogenesis. From this point on, *Xenopus* oocytes are opaque by the presence of cortical granules and pre-melanosomes that begin to appear in the oocyte cortex. During late

stage I-early stage II (200-300 µm diameter), components of the mitochondrial mass, including mitochondria, germ plasm, and maternal RNAs disperse to the future vegetal pole of the oocyte with a dense, apparently disordered, network of MTs fills the oocyte cytoplasm.

Stage III (400-500 µm): Stage III oocytes are characterized by the onset of pigmentation, which gives them a grey color. Pigment is equally distributed in the cortex, and stage III oocytes retain an unpolarized appearance. Lampbrush chromosomes reach their peak near the end of stage III (Gard et al.1995c) [146].

Stage IV (0.5-1.0 mm): Stage IV of oogenesis is marked by the onset of visible polarization of the oocyte along the animal-vegetal (A-V) axis. During this stage, pigment becomes unequally distributed between the cortex of the animal and vegetal hemispheres, resulting in the darkly pigmented animal hemisphere and lightly pigmented vegetal hemispheres characteristic of many amphibian oocytes. A-V polarity is also apparent in the distribution of large yolk platelets, which are concentrated in the vegetal cytoplasm, and the position of the GV, which moves into the animal cytoplasm. Lampbrush chromosomes and nucleoli are concentrated in the center of the GV.

Stage V (1.0-1.2 mm): During stage V, the A-V polarity of the oocyte continues to develop. GV moves into the vegetal cytoplasm and a thin band of basophilic, yolk-free cytoplasm forms near the basal (vegetal) surface of the GV. A sharp equatorial boundary exists between the pigmented animal cortex and the vegetal cortex. Stage V oocytes will undergo meiotic maturation in response to progesterone.

During stage IV-V, the oocyte MT array become progressively more polarized along the animal vegetal axis. In the animal hemisphere, MTs are acetylated and radially-oriented whereas in the vegetal cortex appear less ordered, and fewer are acetylated. Numerous MTs are also apparent in the perinuclear cap of yolk-free cytoplasm found at the base of the GV (Gard et al.1995c) [146].

Stage VI (1.2-1.3 mm): Fully-grown stage VI *Xenopus* oocytes often exhibit a light "equatorial band" separating the pigmented animal and un-pigmented vegetal cortex. The vegetal surface of the GV appears convoluted, and is bounded by a cap of basophilic yolk-free cytoplasm.

e) Confocal microscopy: protocol.

B.bufo tadpoles coming from our laboratories, were previously anesthetized with ethylene glycol monophenyl ether (Merk), then dissected in K Pipes buffer, pH 6,8. Fixation for immunofluorescence microscopy requires a compromise between optimal preservation of cellular structures and preservation of antigenicity and antibody reactivity. According to Becker and Gard,2005 [118] explanted gonads were fixed in formaldehyde-glutaradehyde-taxol (FGT= 3,7% formaldehyde+0,25% glutaraldehyde+0,5 µM taxol) in a fix buffer containing 80 mM K pipes+ 1mM $MgCl_2$+5mM EGTA+0,2% Triton X-100, pH 6,8 for three hours at room temperature (RT), washed in PBS saline buffer (128mM NaCl; 2 mM KCl; 8 mM Na_2HPO_4; 2 mM KH_2PO_4), pH 7,2 and embedded in 5% agar. Section of about 80 µm thickness, obtained using a semiautomatic Vibratome (1000 Classic) were postfixed in methanol (50% 5-10 min, 75% 5-10 min, 80% 5-10 min, 100% 15 min), and rehydrated in TBS saline buffer (10 mM Tris-HCl+155mM NaCl), pH 7,4 for an hour. Subsequently, sections were treated with 75 mM sodium borohydride (SBI) in TBS, pH 7,4 for 1 hour. This step is useful to eliminate the excess of unreactive aldheyde reducing the typical associated auto fluorescence. After washing for 1 hour in TBS, pH 7,4 sections were washed rapidly in PBS, pH 7,4 and then treated with 0,01% pronase in PBS, pH 7,2 for 18 minutes RT. This is an enzymatic reaction useful to unmask the antigenic sites, facilitating the reaction with the antibodies. Sections were washed in TBS+0,1%Triton X-100, pH 7,4 for 1 hour, then permeabilized in TBS+1%Triton X-100, pH 7,4 over night at 4°C. This step is useful to optimized the penetration of the antibodies trough the plasmatic membranes. For the immune localization of the α-tubulin, sections were incubated with a monoclonal anti-α-tubulin antibody produced in mouse (DM1A from Sigma), diluted 1:250 in TBS+0,1% Triton X-100+2% BSA, pH 7,36 for four days at 4°C . After washing with TBS+0,1%Triton X-100 for 8 hours, section were incubated with an anti-mouse polyclonal antibody, conjugated to N,N'-(dipropil)-tethramethyllindocarbocianin (Cy3) (Zymed), diluted 1:150 in TBS+0.1%Triton X-100+ 2%BSA, pH 7.36 for three days at 4°C. Section were washed in TBS+ 0,1% Triton X-100 for about 6 hours. After washing, we have utilized an additional procedure by staining the section with a chromosome dye, 1mM TO-PRO3 iodide (Molecular Probes) in PBS, pH 7,2 for 12 minutes at room temperature and washed in PBS pH 7,4 for few minutes. Section on slides were drained and finally mounted in 2,5% DABCO (Sigma)+90% glycerol (DGT)+50mM TBS, pH 8 90% glycerol to preserve against photobleaching. Slides were stored at 4°C until observation.

Sections are been observed utilizing a Leica TCS SL confocal laser scanning microscopy (CLSM), with Ar/He/Ne lasers and by employing Leica confocal softwate (LCS).

f) Results.

Control sample incubated only with Ab I (DM1A)

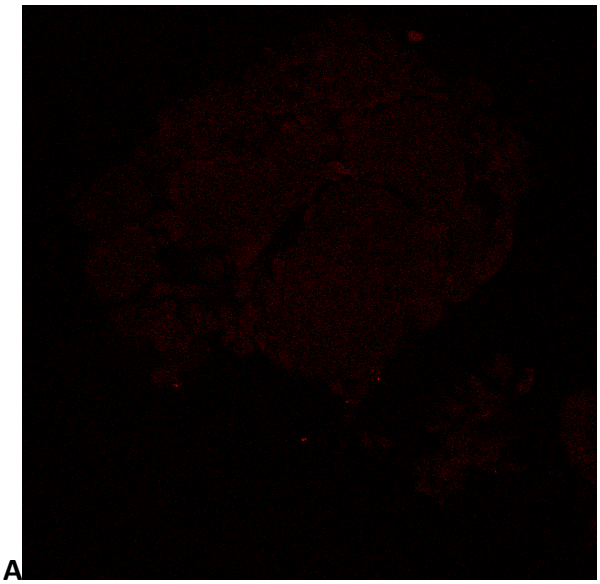
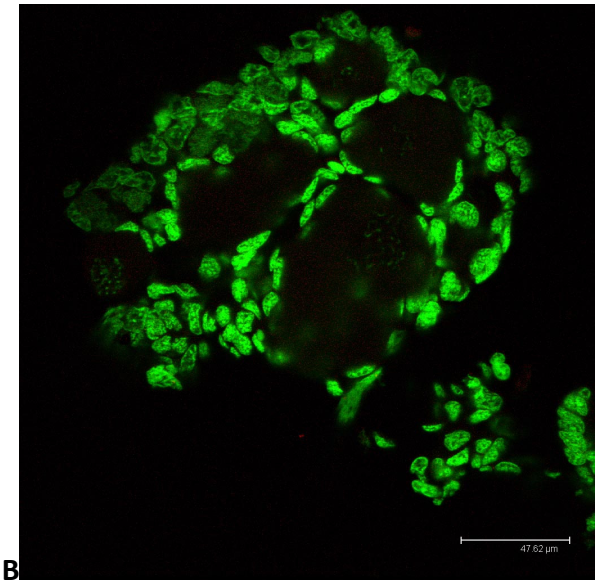
A  B 

Figure 3.13: control sample stained only with Ab I. The background is due to the typical auto-fluorescence of not reactive aldheyde of the fix buffer. Picture A: (channel 0) α-tubulin, (channel 1) nuclei. Picture B: overlay. Scale bar 47,62µm.

Control sample incubated only with Ab II

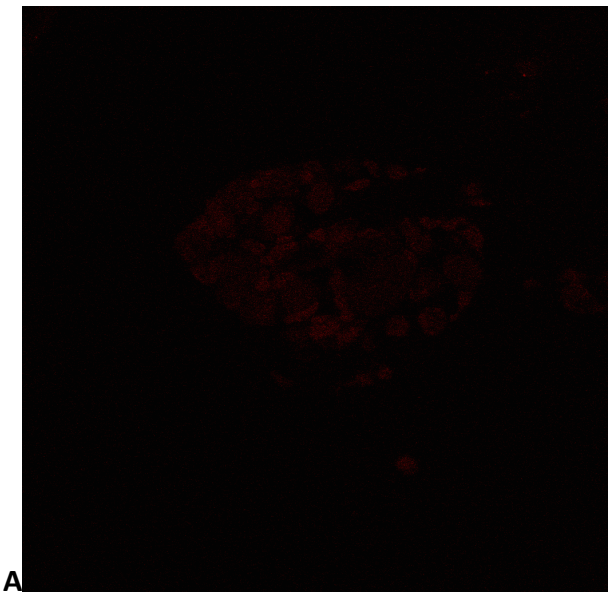
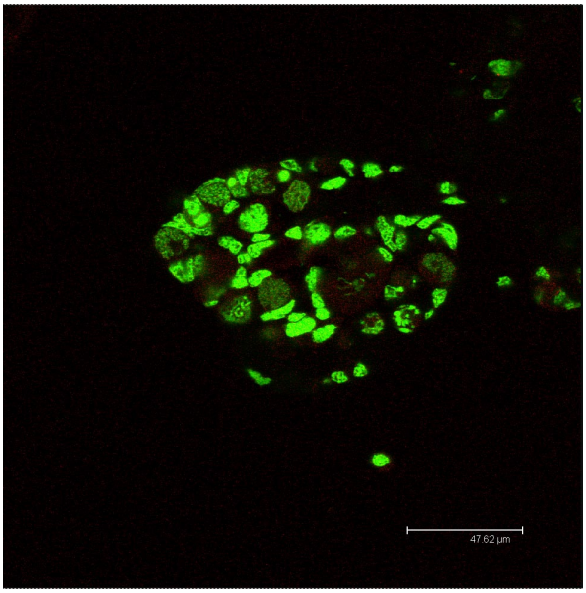
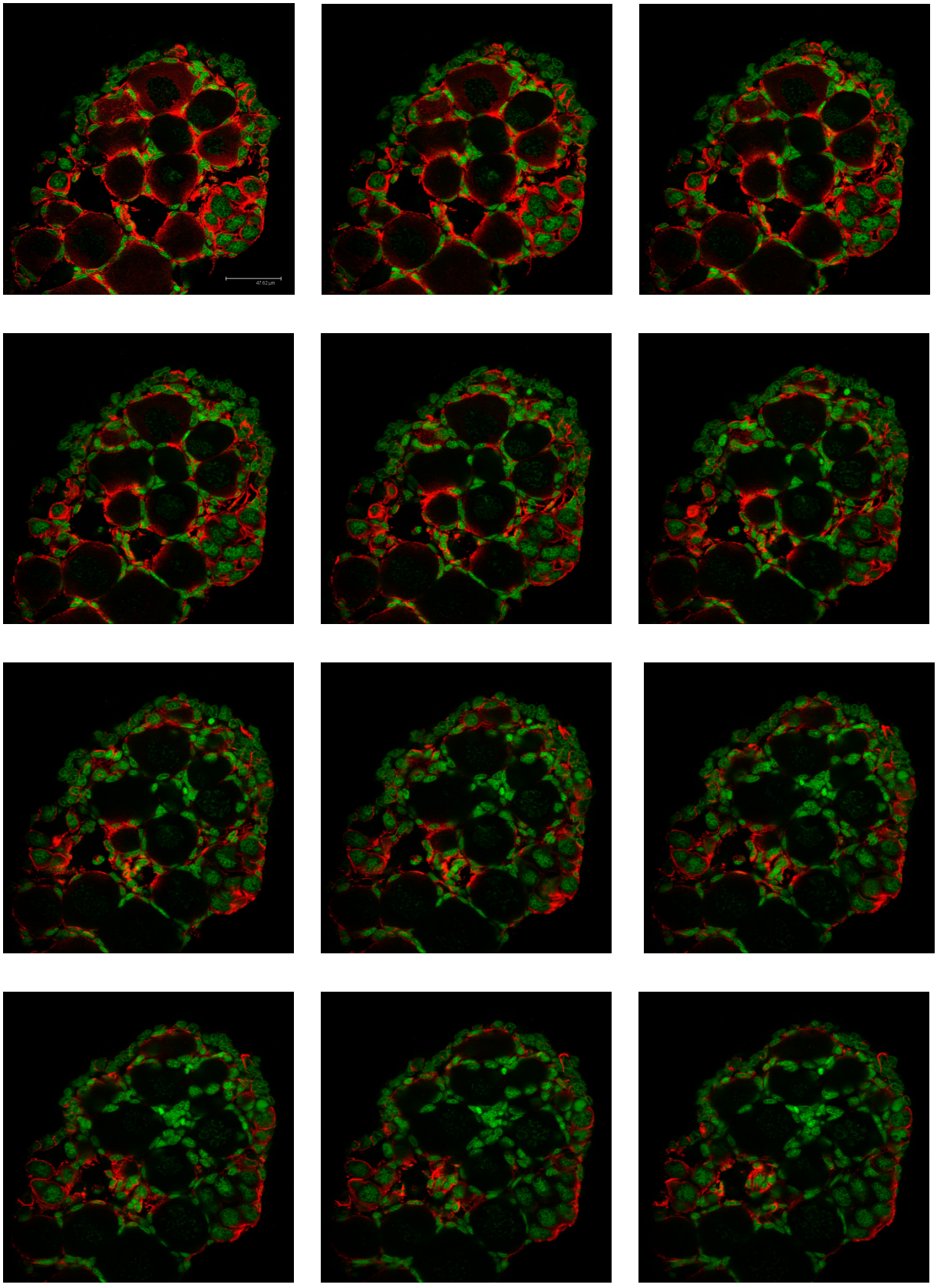
A  B 

Figure 3.14: control sample stained only with Ab I. The background is due to the typical auto-fluorescence of not reactive aldheyde of the fix buffer. Picture A: (channel 0) α-tubulin, (channel 1) nuclei. Picture B: overlay. Scale bar 47,62µm.

Distribution of α-tubluin in the Bidder's organ (female sample).



Series 1: **optical sections of Bidder's organ**. Overlay: channel 0, α-tubuline; channel 1, nuclei. Scale bar 47,62µm

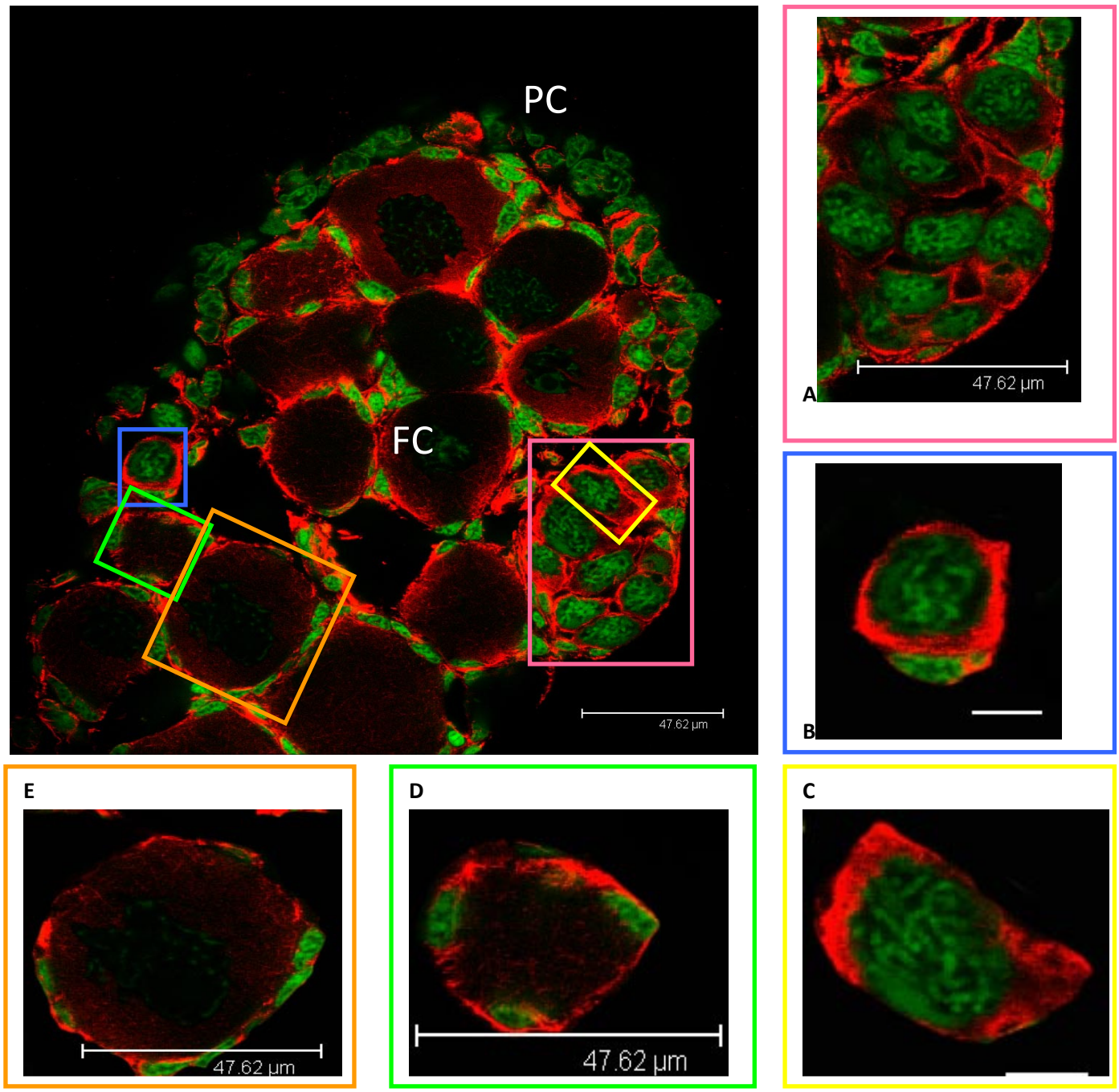


Figure 3.15: Bidder's organ. optical section of the Bidder's organ at stage IV17 showing the typical bipartite architecture (PC=peripheral compartment, FC=follicular compartment). Pictures A,B,C=synaptic oocytes (stage 0), D,E diplotenic oocytes. Overlay: channel 0, α-tubuline; channel 1, nuclei. Scale bar in pictures B,C =9,5 µm

Optical sections reveal the typical ovarian architecture of the Bidder's organ. It present a bipartite architecture consisting in a peripheral compartment, assuming the meaning of germinal epithelium, composed by germ cells in different developmental stages (stage 0 oocytes A,B,C) and a follicular compartment composed by large auxocyte-like diplotenic oocytes (D,E) surrounded by a characteristic mono layer of somatic cells deriving from the basal lamina. Difference in size of diplotenic oocytes (stage I) are related at the two bidderian oogenetic waves that begin in two cronologiccally distinct developmental satges. Bigger oocytes (E), derived from the first bidderian oogenetic wave during stage IV9, whereas smaller oocytes (D) derived from the second bidderian oogenetic wave during stage IV17.

The observations of of Bidder's organ in female and male samples don't show any sostantial difference in the morphological organization as confirm of the rudimentary ovary nature of Bidder's in both males and females.

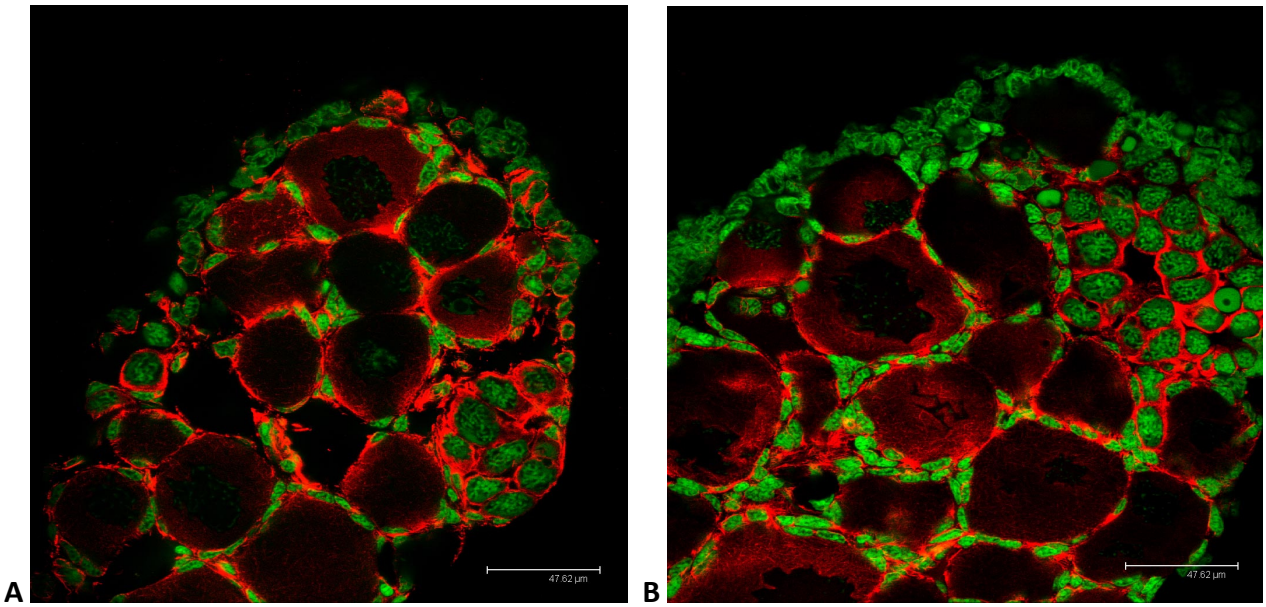


Figure 3.16: Bidders'organ in female (A) and male specimen (B). Overlay: channel 0, α-tubuline; channel 1, nuclei. Scale bar 47,62 µm.

Stage 0 oocytes:

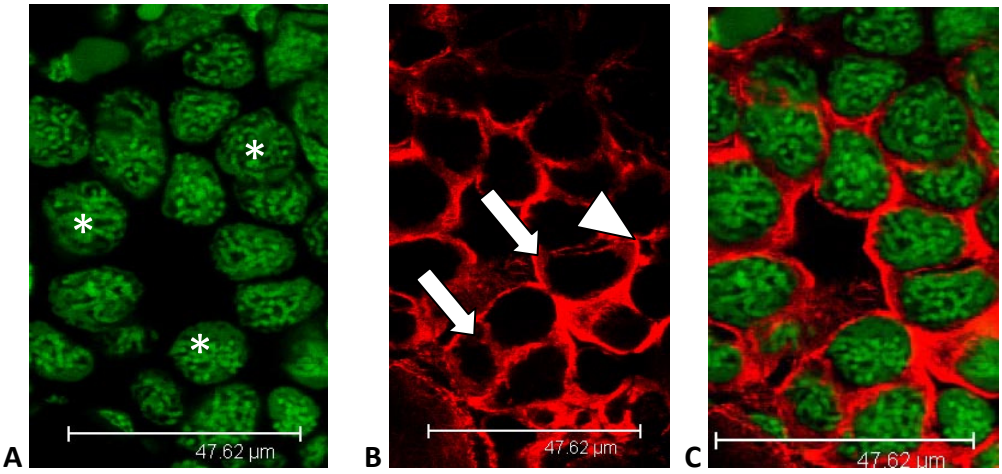


Figure 3.17: stage 0 ooctye. These images are referred to the Bidder organ of male sample. Overlay: channel 0, α-tubuline; channel 1, nuclei Asters=bouquet organization, Arrows=nests, Heads'arrow=MTs concentrated in cap.

Confocal immunofluorescence microscopy revealed that stage 0 oocytes are organized in nests, and contain a dense array of MTs concentrated in the cap of these ovoidal cells with the large, nucleus at the distal end of the cell. As oocytes complete their final S-phase and enter the classic, recombination stages of meiotic prophase (leptotene->zygotene->pachytene), the oocyte chromosomes condense into the classic "bouquet" organization.

***Stage 1 oocytes*:**

Pictures below are rapresentative of stage 1 oocytes characteristics in MTs distribution. Pictures are acquired from the examine in confocal laser scanning microscopy of Bidder's organ of different samples.

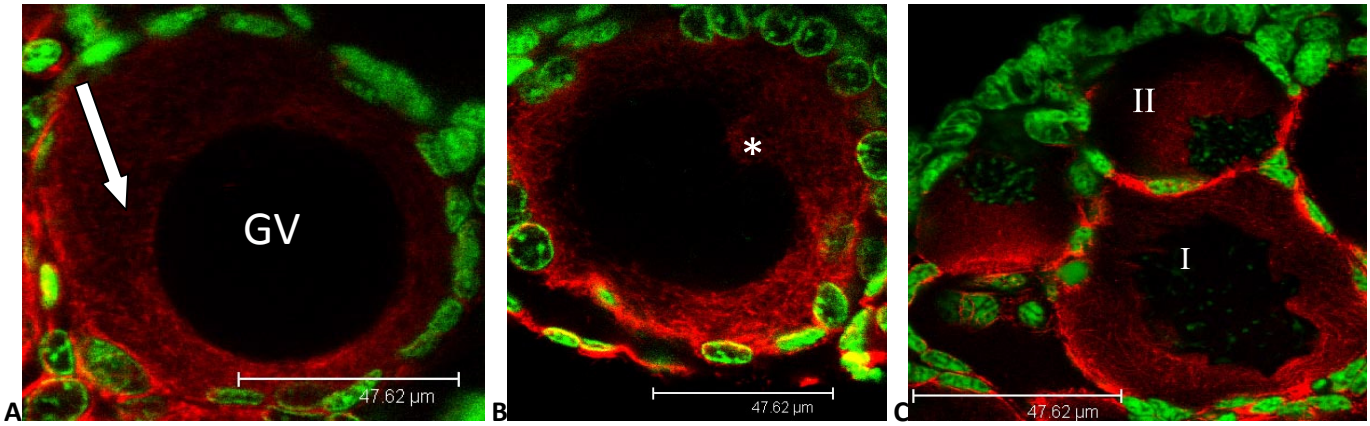
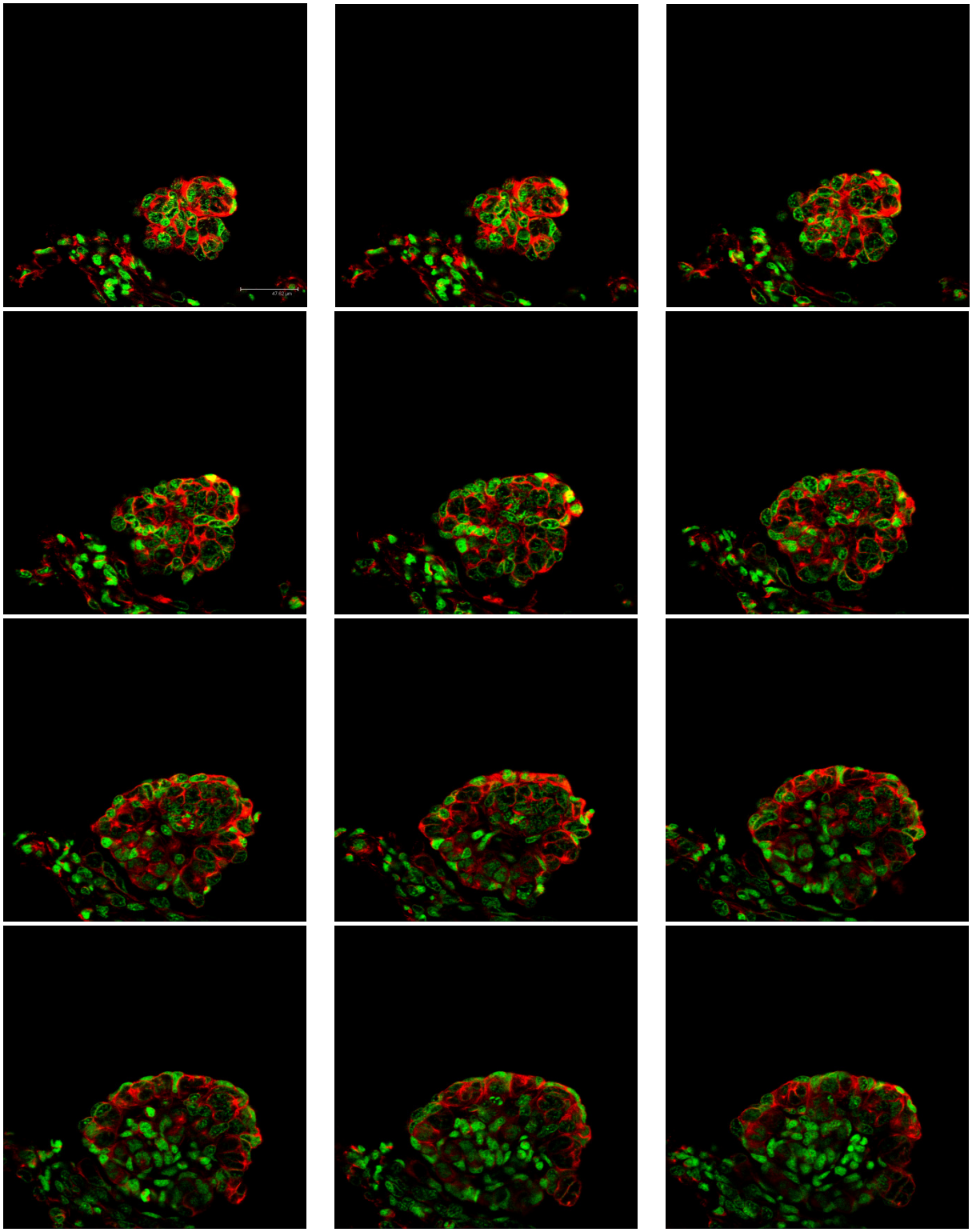


Figure 3.18 stage I oocyte in Bidder's organ. A: diloptenic oocyte of the first oogenetic wave with round germinal vescicle (GV) and MTs dispersed throughout the cytoplasm (arrow), B: diplotenic oocyte of the first oogenetic wave with MTs associated with the mitochondrial cloud (MC), C: diplotenic oocytes of the first (I) and second (II) bidderian vawes. Overlay: channel 0, α-tubuline; channel 1, nuclei. Scale bar 47,62 µm.

Distribution of α-tubluin in the female gonad.



Series 2: **optical sections of a female gonad**. Overlay: channel 0, α-tubuline; channel 1, nuclei. Scale bar 47,62µm

Pictures below are rapresentative of MTs distribution in female gonads. Pictures are acquired from the observation in confocal laser scanning microscopy of different samples.

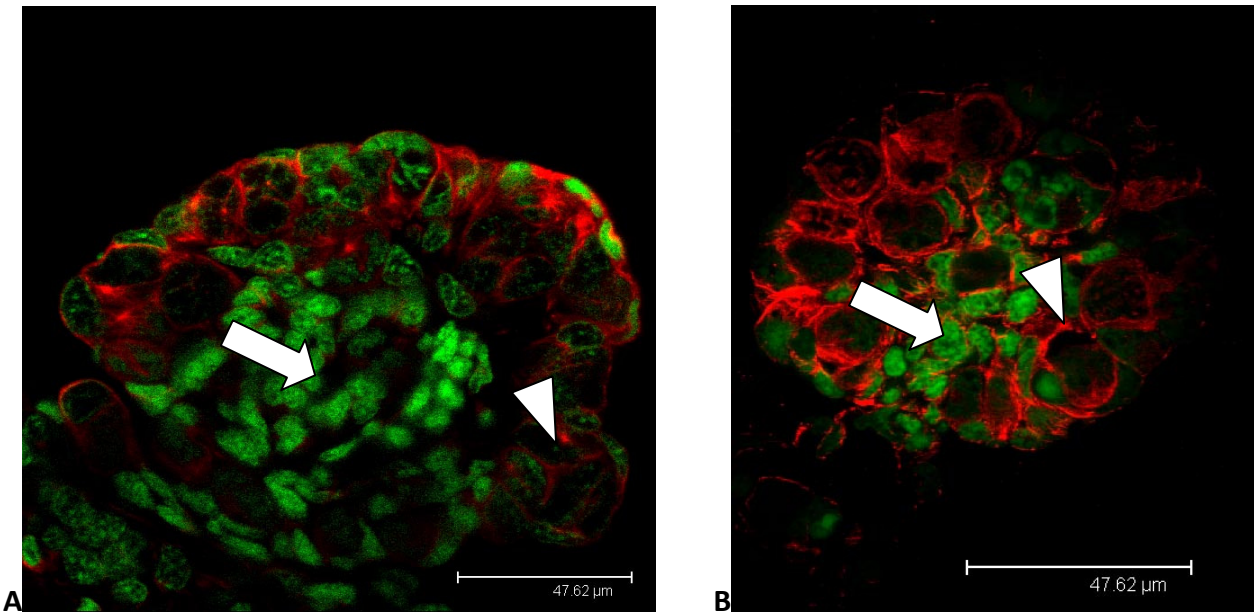
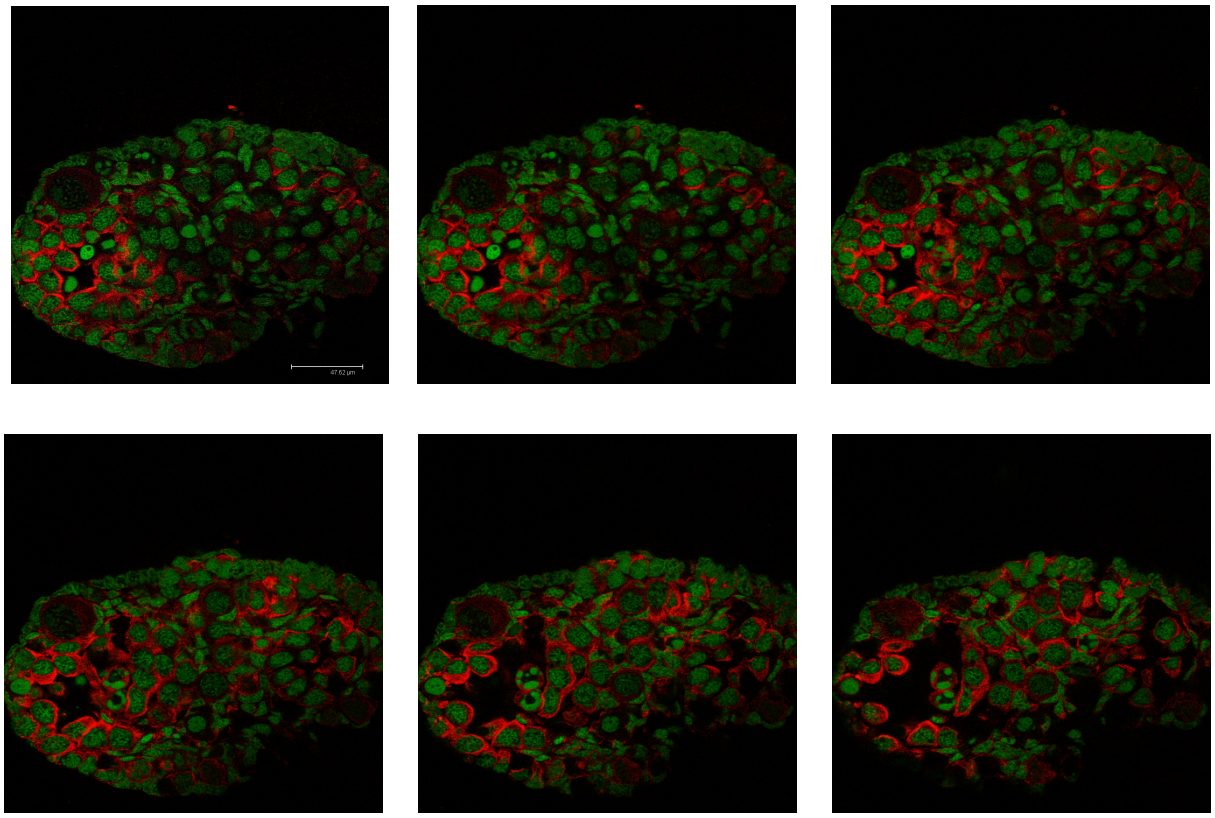


Figure 3.19:female gonad at stage IV17. Pictures A and B are different section examined in different samples. MTs distribution reveal the bistratificate architecture of the female gonads. Germ cells in the cortical layer (head arrows), somatic cells in the medulla (arrows). Overlay: channel 0, α-tubuline; channel 1, nuclei. Scale bar 47,62µm

The general scheme in the morphological architecture of the female gonad at the complement of metamorphosis (stage IV17) by immunofluorescence assay is represented in the pictures above (A,B). Female gonads present a typical bipartite subdivision as underline by the MTs organization: a cortical layer composed by the germinal cells (oogonia), and a medulla composed by somatic cells. Germ cells and medullar somatic cells don't show any morphological interaction: they have not a functional role in the gonadal differentiation.

Depending on development of samples, at the complement of metamorphosis (stage IV17), medullar cells are restricted to a mono layer of somatic cells in defining the incipient ovarian cavity.

Distribution of α-tubluin in the male gonad.

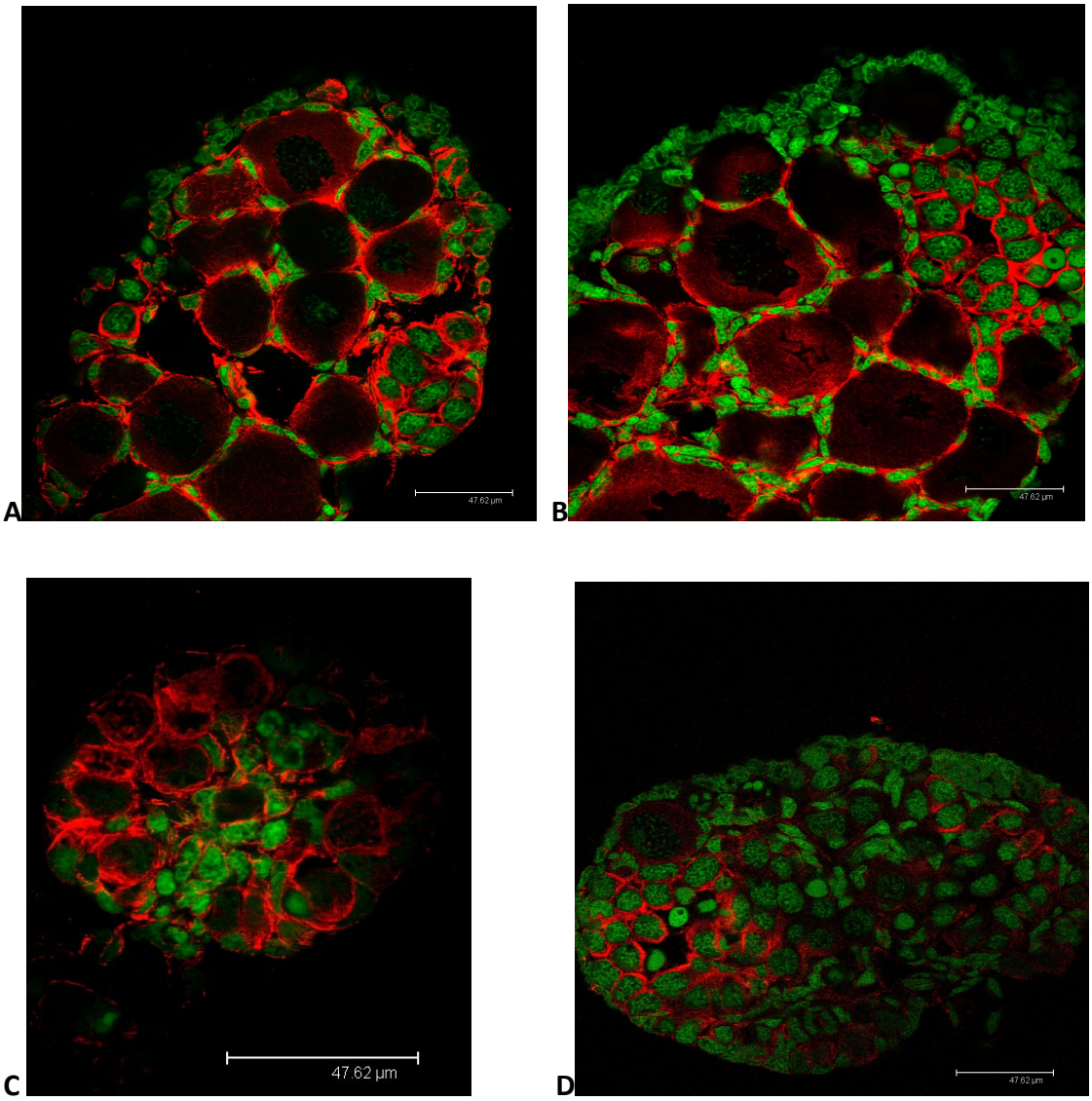


Series 3: optical sections of male gonad. Overlay: channel 0, α-tubuline; channel 1, nuclei. Scale bar 47,62µm.

Figure 3.20:**male gonad at stage IV17**. MTs distribution reveal architecture of the male gonad. Germ cells are inside the medulla (head arrows) with the somatic cells in the medulla (arrows). Overlay: channel 0, α-tubuline; channel 1, nuclei. Scale bar 47,62µm

The general scheme in the morphological architecture of the male gonad at the complement of metamorphosis (stage IV17) by immunofluorescence assay is represented in the pictures above. The morphological evidences in the MTs distribution of testicular development is represented by the fusion of the medulla with the peripheral compartment as result of the GCs centripetal migration.

g) Distribution of α-tubulin in Bidder's organ and in the gonads: discussion.



Figures 3.21 :pictures resuming the MTs distribution in Bidders'organ and in the alternative gonadal architectures. Morphological organization of MTs in female (A) and male (B) Bidder's organ (B), in female gonad (C) and in male gonad (D) at the complement of metamorphosis. Overlay: channel 0, α-tubuline; channel 1, nuclei. Scale bar 47,62µm

Optical sections reveled difference in the MTs distribution in the alternative gonadal phenotypes (stage IV17) and a similar structure of the Bidder's organ in male and female specimen. The result can be summarized as follow:

- Bidder's architecture is similar in males and females, as confirm of the ovarian character of the organ itself (A,B). It consist of a peripheral compartment enveloped by a cortical layer of flattened somatic cells and of a follicular compartment. The peripheral compartment assumed the meaning of a germinal epithelium, because of the presence of germ cells isolated (gonia), in mitotic multiplications (secondary gonia) [not show] and in the first period of meiotic growth (stage 0 from leptotene to pachytene); entering into long diplotenic phase oocytes tend to isolate, each one wrapped by somatic cells, and begin to escape from the peripheral compartment towards the primary cavity to the follicular compartment. Follicular compartment present a typical ovarian organization which

consists of diplotenic oocytes in the second period of growth (auxocytosis) that are leaked by the peripheral compartment to the primary cavity, wrapped individually by a follicle of somatic cells (follicular cells). This mechanism takes place with the formation in the peripheral tissue of a sort of hernia within the primary cavity. The growth of the oocyte, during the auxocitosis period, induce the cell to "fall in" the cavity with an almost passive mechanism. Somatic cells that accompany the oocyte in this movement seem to be derived all from the peripheral compartment: there is not an interruption in the peripheral compartment to allow at the somatic medullar cells to participate in the construction of the follicle. Diplotenic oocyte (stage I) differ in size as a result of the two different bidderian oogenetic waves that begin during chronologically different developmental stages. Oocytes of the first oogenetic wave (stage IV9) are bigger in size if compared to oocyte of the second oogenetic wave (stage IV17).

- the organogenesis of the ovary in genetic females appear as a conservative pathways that substantially maintains the previous undifferentiated architecture. Germ cells are located in the cortex whereas the somatic cells constitute both an external envelope delimiting the gonad (external somatic cells) that a compact medulla (internal somatic cells). During development, the medulla is reduced to a monolayer of cells delimiting the ovarian cavity. Medullar somatic cells have not direct interactions with the germinal elements (C); we can assume that they have not a functional roles in the female sexual differentiation;
- the organogenesis of the testicle seems to happen as a kind of sexual inversion with germinal cells located in the medulla as a consequence centripetal migration as shown in picture D. The translocation of the germinal elements from the cortex to the medulla is a rapid process that happens during stage IV13-IV14 that is responsible of the germ cell-medullar somatic cell interaction. This different interaction between somatic cells and germ cell in the alternative developmental pathways could be correlated with a somatic functional roles of somatic cells in male sexual differentiation.

h) Immuno-detection and localization of Vasa protein in B.bufo samples (stage IV17) by confocal laser scanning microscopy.

i) Function and regulation of vasa gene in different organisms.

The *vasa* gene, essential for germ-cell development, was originally identified in *Drosophila,* to screen the components of the germinal plasm (Lasko et al. 1988) [119] and has since been found in other invertebrates and vertebrates. Analysis of these *vasa* homologues genes has revealed a highly conserved role for Vasa protein among different organisms, as well as some important differences in its regulation.

The *vasa* gene encodes an ATP-dependent RNA helicase of the DEAD-box family and is required for promoting translation of at least two known mRNAs, *nanos* and *gurken* in *Drosophila*. Vasa RNA is broadly distributed in Drosophila eggs, however Vasa protein is a component of germ plasm and is required for normal development of the PGCs. VASA proteins are found in primordial germ cells (PGCs) in species ranging from hydra to humans.

The distribution of *vasa* RNA or protein was determined during different stages of development in different organisms, thus providing information on the possible function of *vasa* during germ-cell development in these species (Lasko and Ashburner 1990) [120].

Vasa gene homologues have been isolated from several species of teleost fishes, including zebrafish, medaka, tilapia. In zebrafish maternal vasa RNA is a component of germ plasm; however the protein it encodes is not (Knaut et al. 2000) [121]. This pattern is the inverse of vasa expression in Drosophila and is different from Xenopus. There are several possible explanations for this paradox. One is that in zebrafish it is the *vasa* RNA and not the protein that is important for the early determination of germ plasm. Another possible explanation of the paradox is that although most of the Vasa protein is uniformly distributed in all cells, it is active only in the germ cells, as a result of cell-specific post-translational modifications.

Genes homologous to *vasa* have been identified in *Xenopus* (Komiya et al.1994) [122] and *Axolotls* (Drum and Johnson unpublished data). The *vasa* gene shows diverse pattern of expression in these amphibians. In *Xenopus* embryos maternal vasa RNA is found early in germ cells precursor as well as in somatic cells and the zygotic gene does not show cell-specific expression (Ikenishi and Tanaka, 2000) [123]. The protein encoded by the vasa gene, on the other hand, segregates with the germ plasm through the early stages of development (Ikenishi 1998) [124]and is required for PGC development (Ikenishi and Tanaka 1997) [125].

Maternal *vasa* RNA in axolotls is not germ cell specific: it is randomly distributed in early embryos. The zygotic gene is not activated until late in development , much later than in Xenopus, and in a cell specific manner in PGCs in the gonads. The distribution of Vasa protein in axolotls has not yet been investigated.

Finally, in amniotes, chick VASA protein is maternally inherited and segregates with the germ cell from the inception of their development. In mouse embryos vasa RNA and protein are not maternally inherited; they are first expressed in PGCs after they colonize the genital ridge (Fujiwara et al. 1994; Toyooka et al. 2000) [126,127]. Also, homozygotes for a null allele of the mouse vasa gene, *mvh*, demonstrate that *vasa* gene products are not required to produce PGCs.

Rather, *mvh* is required only in male gonadal germ cells to complete meiosis (Tanaka et al.2000) [128]. Therefore, the role of vasa genes from mouse and Xenopus has not been conserved.

The function of the *vasa* gene can be inferred from its expression pattern in different organisms and from phenotypic analysis of animals lacking a functional gene. With the exception of the mouse (and probably other mammals), the *vasa* gene product is expressed in or localized to the PGCs very early in development, consistent with the idea that its activity is required for specification of this cell lineage. Interestingly, in planarians, where a *vasa* homolog is also expressed in the soma, the somatic cells that express the gene were identified as neoblasts - a totipotent cell type that functions in regeneration. The function of *vasa* could therefore be described as important for preserving totipotency. One mechanism for preserving totipotency is to inhibit expression of genes that would lead to somatic differentiation. An indirect role for *vasa* in transcriptional inhibition is suggested by the finding that one of the few known targets of Vasa, *nanos,* can repress gene expression in the *Drosophila* germline . In the mouse, where germ cells are induced through cellular interactions rather than by inheritance of maternal cytoplasmic determinants, the expression of *vasa* is initiated relatively late. Vasa is expressed in the PGCs as they arrive at the gonad, and expression is induced by interaction between the germ cells and the somatic cells of the developing gonad. The expression of *vasa* at this stage in the mouse, as well as in all the other organisms described above, is likely to reflect a requirement for the gene product for differentiation of the germ cells into gametes. Indeed, loss of *vasa* function in the mouse affects differentiation of the male germ cells, resulting in male sterility (no other phenotype is observed in the knockout mice). Similarly, a late function of *vasa* during gametogenesis has been described in the nematode and the fly. The first mechanistic evidence coupling progression in gamete differentiation and *vasa* function is the demonstration that a meiotic checkpoint during oogenesis in *Drosophila* appears to control the activity of the Vasa protein.

j) Confocal microscopy: protocol.

B.bufo tadpoles coming from our laboratories, were previously anesthetized with ethylene glycol monophenyl ether, then dissected in K Pipes buffer, pH 6,8. Fixation for immunofluorescence microscopy requires a compromise between optimal preservation of cellular structures and preservation of antigenicity and antibody reactivity. According to Becker and Gard,2005 [118] explanted gonads were fixed in formaldehyde-glutaradehyde-taxol (FGT= 3,7% formaldehyde; 0,25% glutaraldehyde; 0,5 µM taxol) in a fix buffer containing 80 mM K pipes; 1mM $MgCl_2$; 5mM EGTA; 0,2% Triton X-100, pH 6,8) for three hours at room temperature (TR), washed in PBS saline buffer (128mM NaCl; 2 mM KCl; 8 mM Na_2HPO_4; 2 mM KH_2PO_4), pH 7,2 and embedded in 5% agar. Section of about 80 µm thickness, obtained using a semiautomatic Vibratome (1000 Classic) were postfixed in methanol (50% 5-10 min, 75% 5-10 min, 80% 5-10 min, 100% 15 min), and rehydrated in TBS saline buffer (10 mM Tris-HCl;155mM NaCl), pH 7,4 for an hour. Subsequently, sections were treated with 75 mM sodium borohydride (SBI) in TBS, pH 7,4 for 1 hour. This step is useful to eliminate the excess of unreactive aldheyde reducing the typical associated auto fluorescence. Sections were washed in TBS+0,1% Triton X-100, pH 7,4 for 1 hour, then permeabilized in TBS+1% Triton-X 100, pH 7,4 over night at 4°C. This step is useful to optimized the penetration of the antibodies trough the plasmatic membranes.

Non specific protein binding sites were blocked in TBS+0,1%Triton X-100+2% BSA+1% normal goat serum (NGS) (BSA and NGS from Sigma), pH 7,4 for 1 hour at room temperature before the incubation with primary antibody, an antiserum against a chicken Vasa protein (CVP), produced in a rabbit (a gift from Noce), diluted 1:1500 with TBS+0,1% Triton X-100+ 2% BSA+ 1% NGS, pH 7,3 for four days at 4°C. After washing with TBS+ 0,1% Triton-X 100 for 8 hours, section were incubated with an anti-mouse polyclonal antibody, conjugated to N,N'-(dipropil)-tethramethyllindocarbocianin (Cy3) (Zymed), diluted 1:150 in TBS+ 0,1% Triton X-100+ 10%NGS+ 2%BSA for three days at 4°C. Section were washed in TBS; 0,1% Triton X-100 for about 6 hours. After washing, we have utilized an additional procedure by staining the section with a chromosome dye, 1mM TO-PRO3 iodide (Molecular Probes) in PBS, pH 7,2 for 12 minutes at room temperature. Subsequently, sections were washed in PBS pH,7,4 and finally mounted in slides (previously treated with EtOH 70%+1%HCl) by using a solution 2,5% DABCO (Sigma);50mM Tris, pH 8, 90% glycerol (DGT) as anti-fading. Slides were stored at 4°C until observation.

Sections were observed utilizing a Leica TCS SL confocal laser scanning microscopy (CLSM), with Ar/He/Ne lasers and by employing Leica confocal softwate (LCS).

k) Results.

Control sample incubed only with Ab I

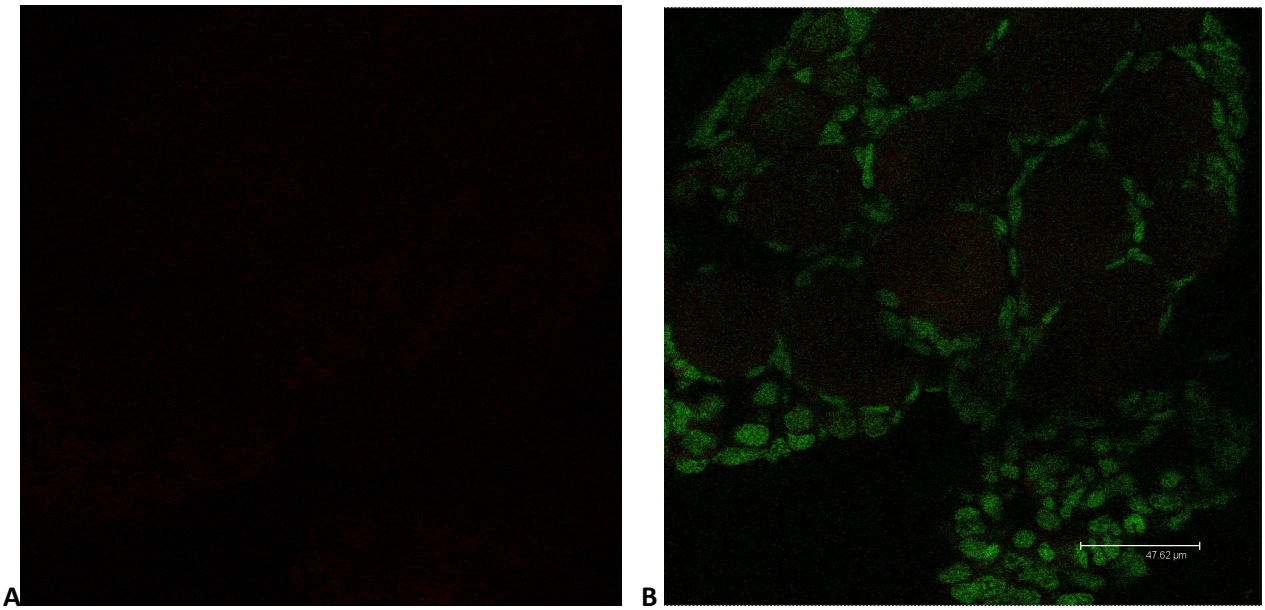


Figure 3.22: control sample stained only with Ab I. The background is due to the typical auto-fluorescence of not reactive aldheyde of the fix buffer. Picture A: (channel 0) Vasa, (channel 1) nuclei. Picture B: overlay. Scale bar 47,62µm.

Control sample incubed only with Ab II

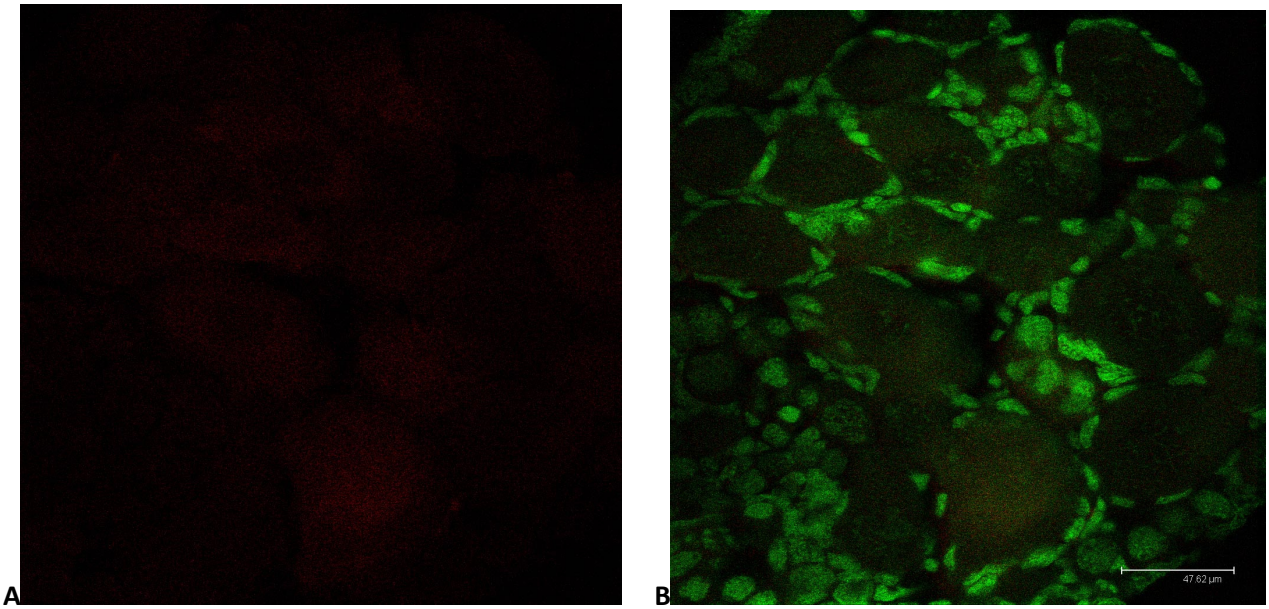


Figure 3.23: control sample stained only with Ab I. The background is due to the typical auto-fluorescence of not reactive aldheyde of the fix buffer. Picture A: (channel 0) Vasa, (channel 1) nuclei. Picture B: overlay. Scale bar 47,62µm.

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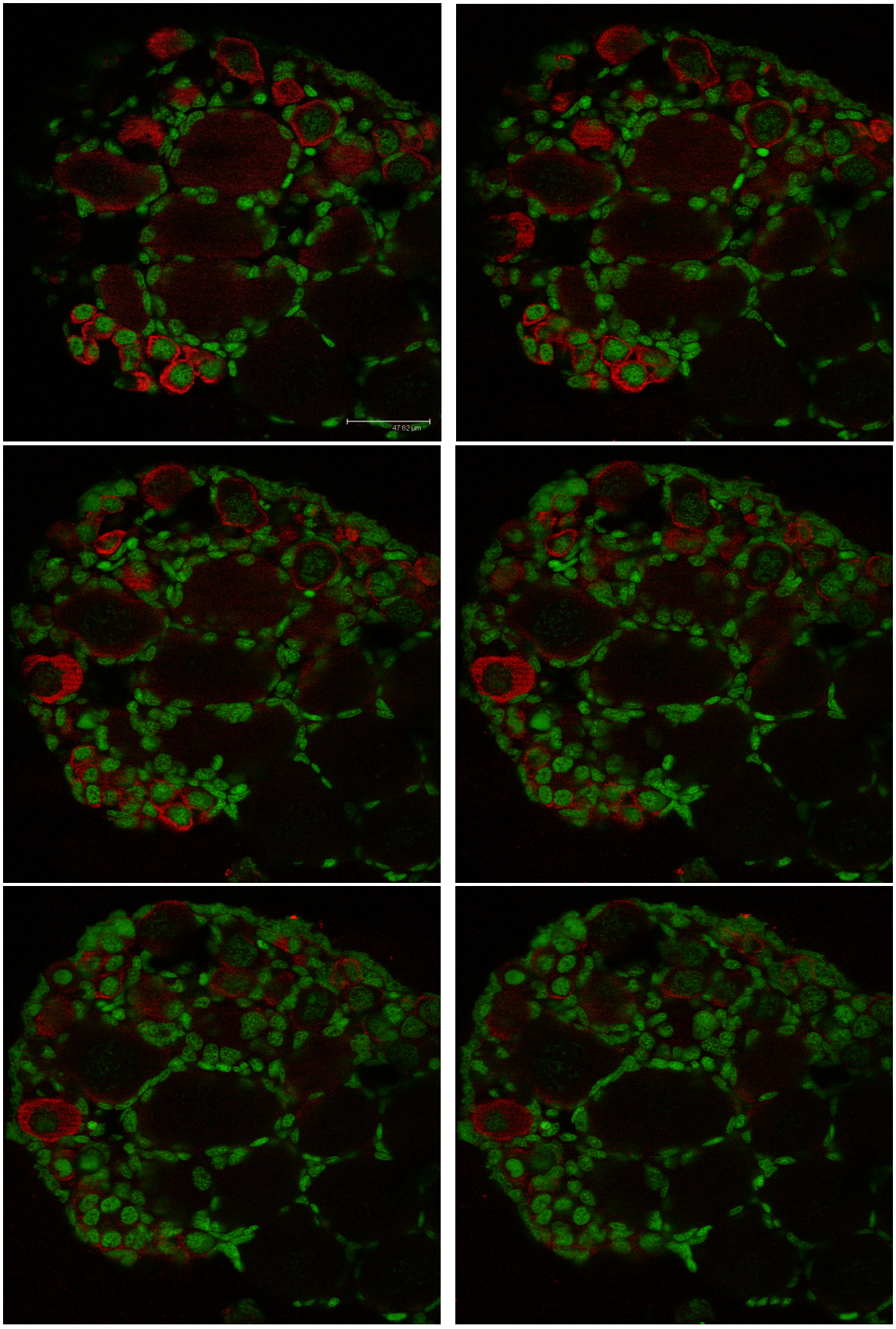
Distribution of Vasa protein in the Bidder's organ.



Series 4: **optical sections of Bidder's organ**. Overlay: channel 0, Vasa; channel 1, nuclei. Scale bar 47,62µm

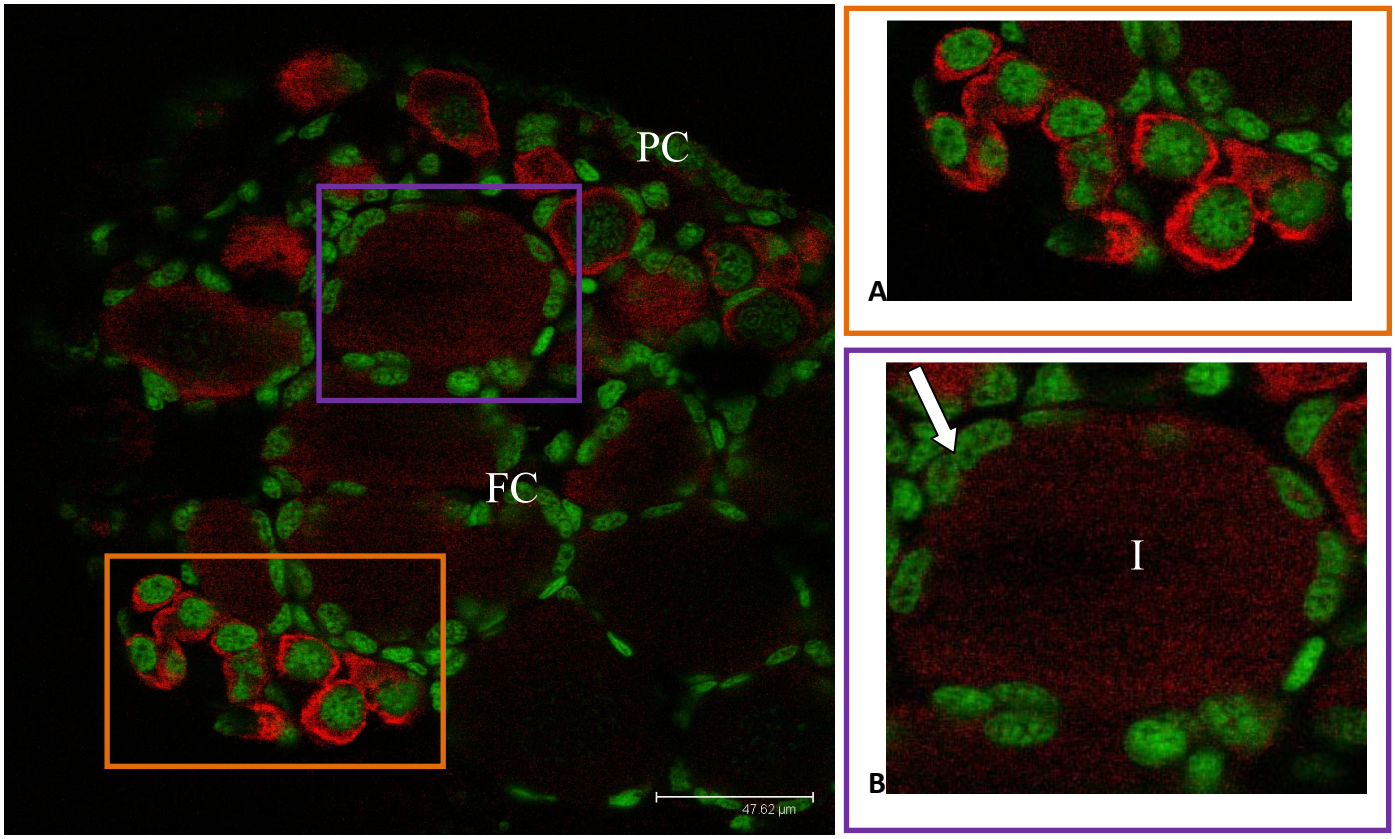


Figure 3.25: Bidder's organ. optical section of the Bidder's organ at stage IV17 showing the typical bipartite architecture (PC=peripheral compartment, FC=follicular compartment). Pictures A =synaptic oocytes (stage 0), B diplotenic oocytes of the first (I)bidderian wave. Overlay: channel 0, Vasa; channel 1, nuclei. Scale bar 47,62 µm

The distribution of Vasa protein detected in Bidder's permits to discriminate in a specific manner the germinal cell (that present the positivity at cytoplasm level) from the somatic cells. The analysis of the germinal elements present different intensity in coloration according to the cellular growth and a consequent distribution of Vasa protein in the cytoplasm.

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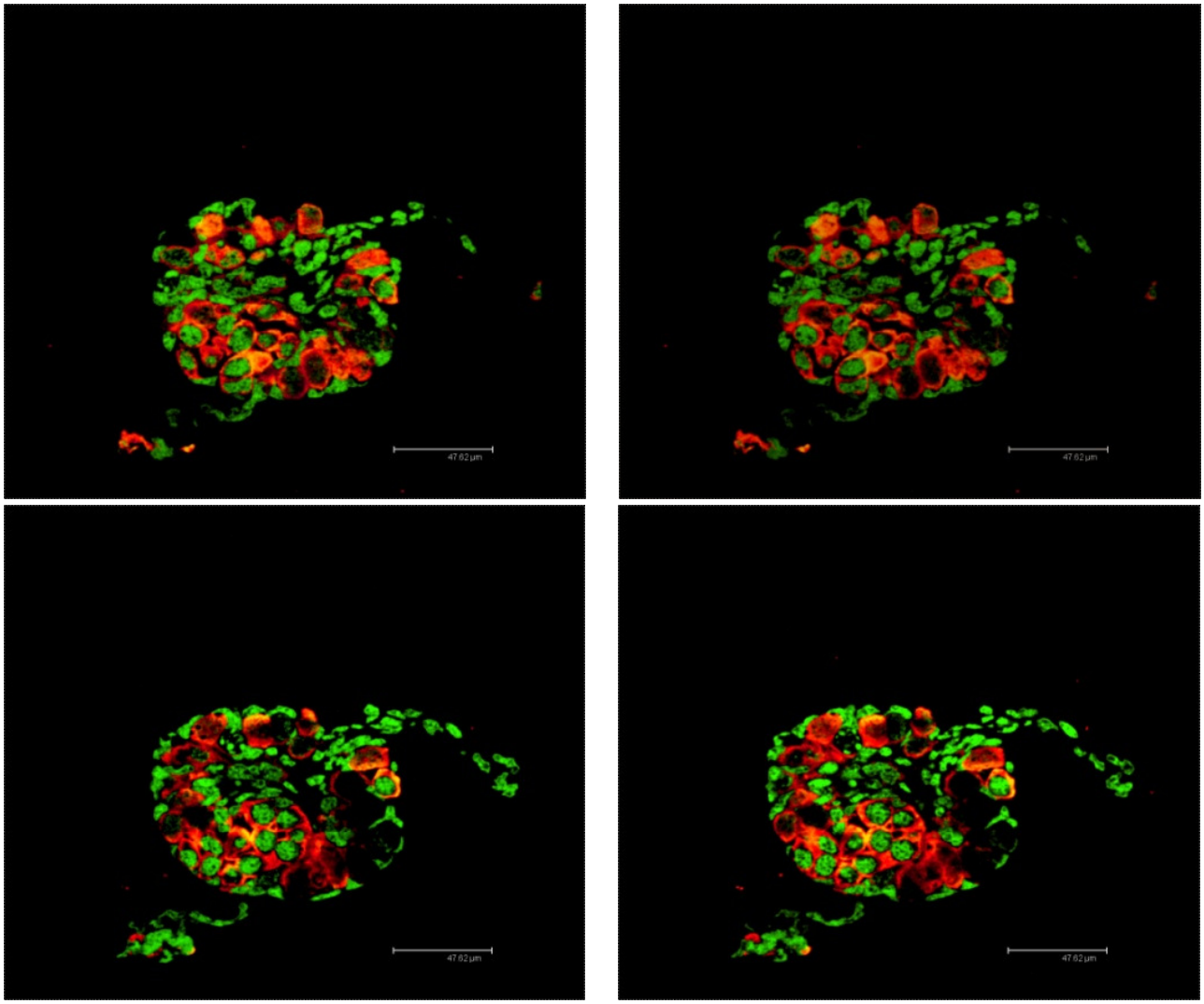
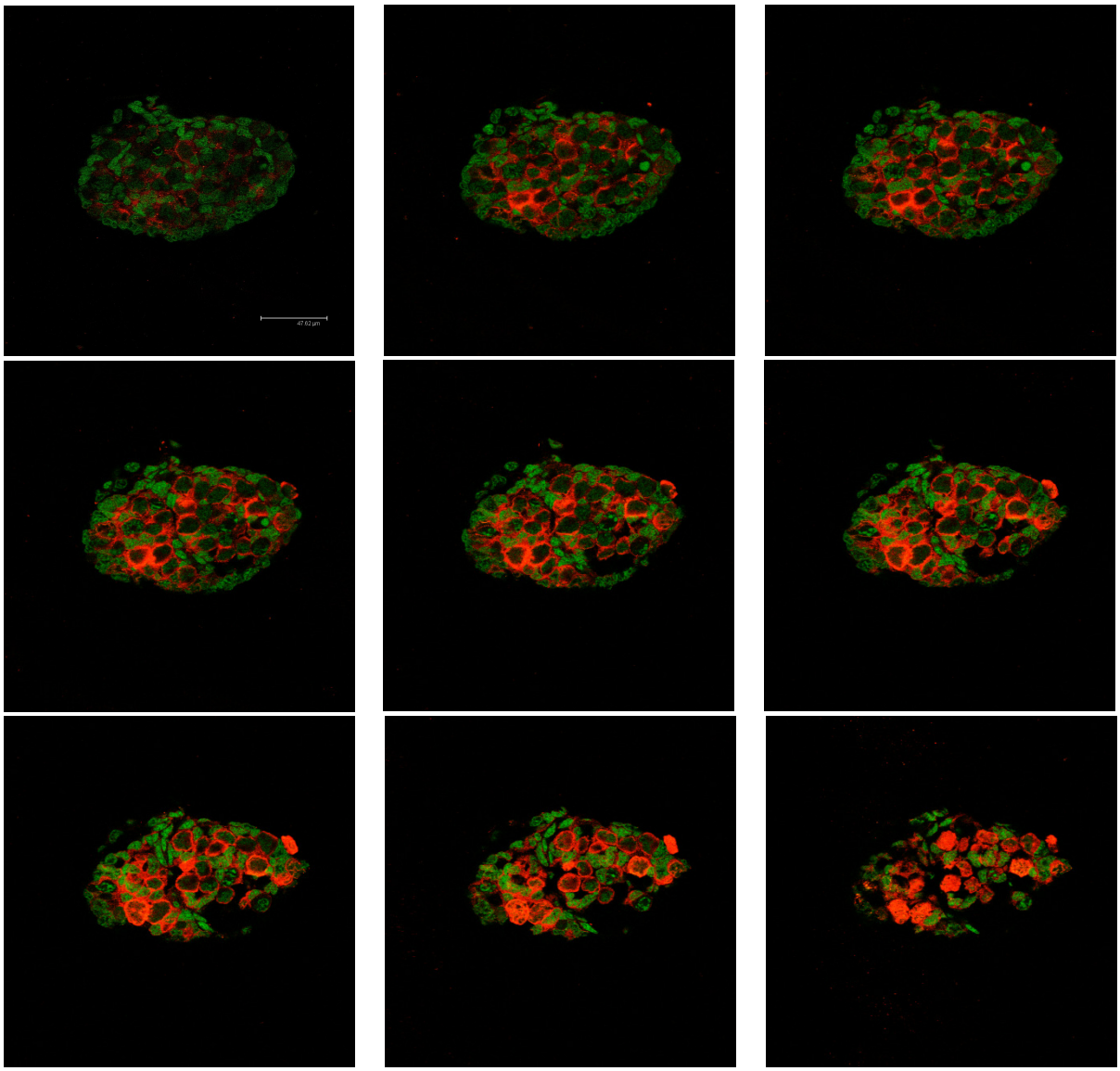
Distribution of Vasa protein in the female gonad.



Series 5: optical sections of female gonad. Overlay: channel 0, Vasa; channel 1, nuclei. Scale bar 47,62µm.

The general scheme in the morphological architecture of the female gonad at the complement of metamorphosis is represented by the spatial distribution of *Vasa* protein in the pictures above (series 5). Female gonads present a typical bipartite subdivision: a peripheral compartment composed by the germinal cells with the typical red coloration *Vasa*-associated and a central compartment (medulla) constituted by somatic. No signal is detect at somatic level as confirm of the specificity of Vasa to detect germ cells also in *B.bufo.*

Distribution of Vasa protein in the male gonad.



Series 6: optical sections of male gonad. Overlay: channel 0, Vasa; channel 1, nuclei. Scale bar 47,62µm.

Series 6 represent the morphological architecture of a typical male gonad. The evidences of an incipient testicular development is represented by the fusion of the medulla with the peripheral compartment as result of the GCs centripetal migration.

l) Spatial distribution of Vasa protein in the Bidder's organ and in the gonads: discussion.

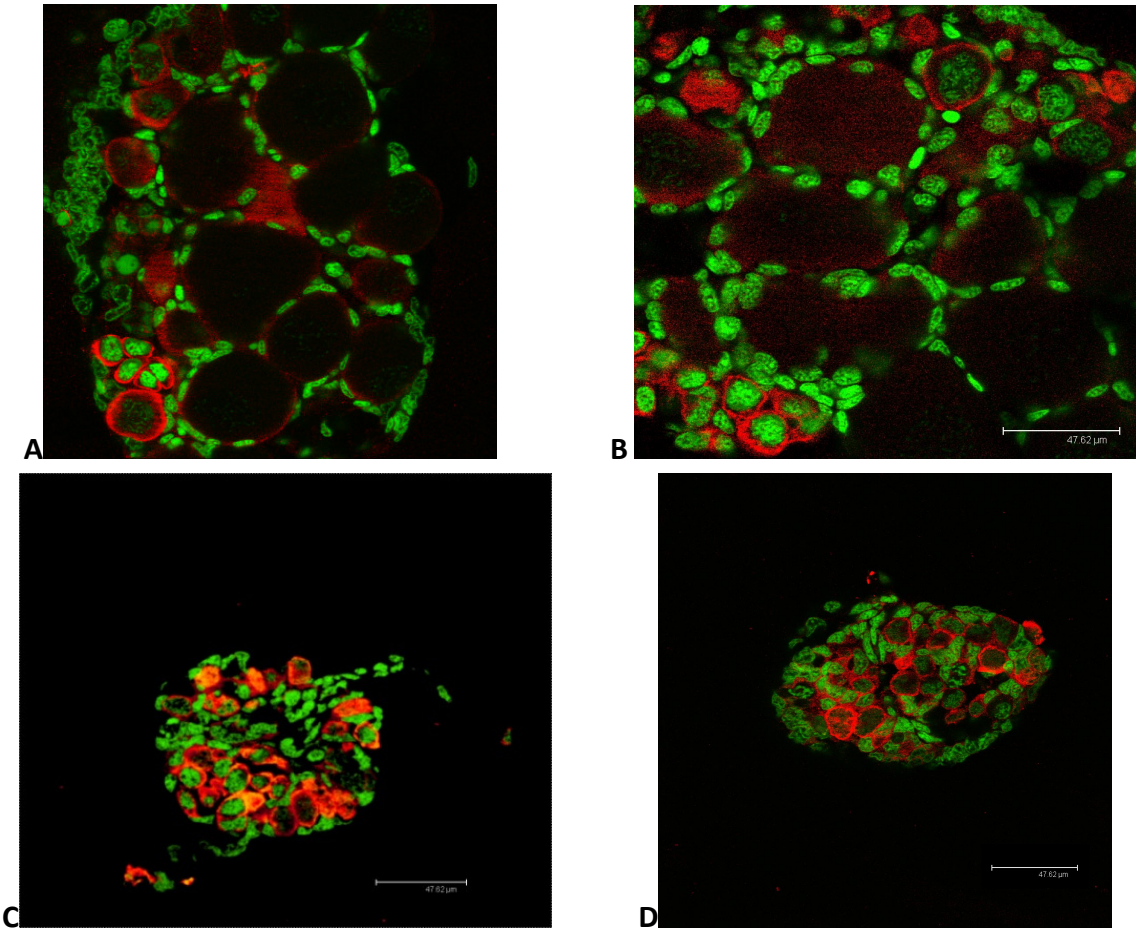


Figure 3.26. Vasa detection in Bidder' organ (A=female; B=male); female gonad (C) and male gonad (D). Overlay: channel 0, Vasa; channel 1, nuclei. Scale bar 47,62µm.

Optical sections reveled difference in the Vasa distribution in the alternative gonadal phenotypes (stage IV17) and a similar structure of the Bidder's organ in male and female specimen. The result can be summarized as follow:

- Bidder's organ present a single pattern, similar in males and females. There are no difference between sexes in the cellular distribution of *Vasa* protein. It's architecture resumed a typical ovarian structure corresponding to the classical description: a peripheral compartment assuming the meaning of a germinal epithelium (composed by germinal elements at early stage of development) and a follicular compartment, composed by diplotenic oocytes. In contrast to α-tubulin, the specific signal of *Vasa* associated at the germinal cells permits an higher resolution it the detection of stage 0 and stage I oocytes. The *Vasa* associated signal in bidderian germinal cells decrease during oocyte differentiation, higher in synaptic oocytes and lower in diplotenic oocytes as a consequence of the cellular growth and a distribution of *Vasa* throughout the bigger cytoplasm;
- the organogenesis of the ovary confirm the evidence listed during the discussion of the distribution of α-tubulin. It consist in a typical bipartite architecture with the germinal cells

located in the cortex and a compact medulla of somatic cells. No germ cells-soma interaction are highlighted; medullar somatic cells have not direct interactions with the germinal elements and not participate in female sexual differentiation;

- also the organogenesis of the testicle confirm the evidence listed during the discussion of the distribution of α-tubulin. The translocation of the germinal elements from the cortex is responsible of the germ cell-medullar somatic cell interaction. This different interaction between somatic cells and germ cell in the alternative developmental pathways could be correlated with a somatic functional roles of somatic cells in male sexual differentiation;
- no difference in the distribution of *Vasa* associated signals are detected in the germinal lineage of males and females gonads as confirm that germ cells at the complement of metamorphosis are in the same developmental phase.

m) Spatiotemporal distribution of Sox9 in the gonad and in the Bidder organ of *B.bufo*.

n) Introduction: Sox9, a marker of male sexual development in vertebrate?

In eutherian mammals, the presence of the Y chromosome-linked SRY (Sex determining Region on Y chromosome) gene is sufficient to promote the testicular development in mammals.
Sry gene has a HMG (high mobility group) DNA-binding domain which is present in some of the classes of non-histonic proteins as the Sox family.
The name of the Sox family derives from the fact that *Sox* genes all share the same HMG box (*SRY*-related HMG-box) which binds to the minor groove of DNA bending and unwinding the DNA double helix through an angle that is characteristic of the various SOX protein. It has generally been assumed that this ability to bend DNA is an absolute requirement for gene transcription.
A number of the *SRY*-related HMG box (*Sox*) genes have been isolated from a wide variety of organisms classified into many subgroups and SOX proteins have diverse functions in various tissues of vertebrates during development judging by the expression of *Sox* genes in different tissue (Marshall et al. 2000) [129].
The SOX9 protein, like SRY, has an HMG box that binds to specific DNA sequences. In addition, SOX9 has two transcriptional activation domains. It is therefore safe to assume that SOX9 protein binds and activates genes in male sex-determining pathway (Koopman et al.2001) [149]. This is analogous to the known role of *Sox9* during chondrogenesis. In chondrogenesis, the mechanism that give rise to endochondral bones, SOX9 involves the activation of a number of collagen gene that compose cartilage such as *Col2a1* (encoding type II collagen) which is the major component of cartilage(Bell et al.1997) [130].
In male development, the requirement of SOX9 for male sex determination in humans is demonstrated by the fact that translocation and mutation affecting *Sox9* results in campomelic dysplasia (CD) (Foster et al,1994 ;Wagner et al,1994) [131,132]. Campomelic dysplasia is a rare, often lethal, genetic condition characterized by multiple abnormalities including short limbs, bowed legs, distinctive facial features, and a narrow chest. It is also often associated with abnormal development of the sex organs in males (associated with XY sex reversal) resulting by the loss of function of only one copy of *Sox9*; it would seem that *Sox9* operate in a dosage-sensitive mechanism.
In mouse, *Sox9* expression is expressed in the genital ridge of both sexes at low levels but only in males persist after *Sry* has peaked*; Sox9* is up-regulated in male genital ridges and a down-regulated in female ones. The possible regulative mechanisms describing the relationship between *Sry* and *Sox9* could be three*: Sry* might directly the up-regulation of *Sox9* in males, *Sry* might up-regulate an intermediate gene inducing the Sox9 expression or SRY might repress a gene that represses Sox9 expression.
Sox9 expression in mammals precedes that of Amh (Kent et al 1996) [133]; it has been established beyond reasonable doubt that SOX9 regulates the expression of Amh gene because of able to bind the Amh promoter. SOX9 act in differentiating the reproductive tract by up-regulating the expression of anti-Mullerian hormone (AMH), a cell specific factor involved in male differentiation of the internal genitalia. The regulation of AMH is complex protein-protein interactions exist

between SOX9 and SF1, SF-1 and GATA-4 and SF-1 and WT1 but the pivotal role in AMH activation is played by SOX9 (De Santa et al.1998) [134].

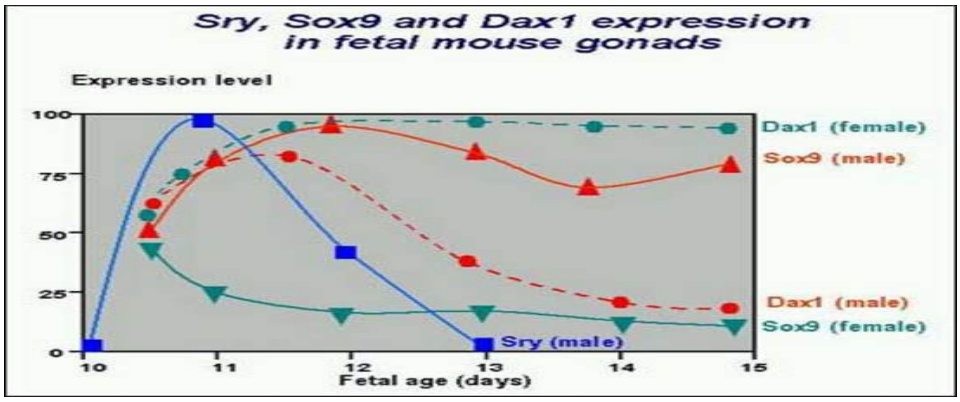


Figure 3.27:temporal expression of Sry, Sox9 and Dax1 in mouse fetal gonads (from Rey).

What is the function of *Sox9* during gonadal development in non-mammalian?

Non-mammalian vertebrates lack a *Sry* gene. *Sox*9 appeared to be the earliest testis-specific gene in chicken.

In the chicken and alligator Amh expression precedes *Sox9* expression (Oreal et al.1998, Western et al.1999) [135,136], implying that Sox9 is not required for the transcription of AMH in these species. Conversely, in turtles *Sox9* appears to follow the mammalian pattern of expression (Moreno-Mendoza et al.1999) [150]. It should be noted that SOX-binding sites do exist in the chicken Amh promoter and considering that the start of *Sox9* expression appears to coincide with a significant increase in chicken AMH expression it is conceivable that Sox9 may be involved in up-regulating AMH in birds at least (Orel et al.1998)[135].

During normal development of the chick, *cSox9* expression is detected in the genital ridge at low level in early stages (stage 25) at which the genital ridges look morphologically identical. *cSox9* is also transiently expressed in mesonephronic tubules, which are functional in the chick, at these early stages. Reaching the first signs of morphological differences between the sexes, *cSox9* expression shows differential pattern: down regulated in ZW gonads and upregulated in ZZ gonads. As in the mouse, the high levels in ZZ gonads preceded overt cord formation. In ZW embryos the expression was down regulated in both the left gonad, which normally develops as an ovary, and in the right gonad which was retained in an indifferent or immature state (da Silva et al.1996)[152].

In the leopard gecko *Eublepharis macularius,* the *gSox9* transcript is present in the genital ridge of the male embryos up to stage 40, the last stage observed. In this gecko species sex is known to be determined irreversibly between stages 32 and 37, the stages in which gSox9 is expressed in all embryos; however, there are no data relating to the morphology of the ovary or testis at this time. As with the other species observed, it is only after testis determination has begun that Sox9 is expressed in a sex-dependent manner (Elizabeth et al.2001)[151].

In teleost fishes *Sox9* is present in two different forms called *Sox9a* and *Sox9b* expressed in chondrogenic tissues. In the zebrafish *Sox9a* is expressed in the testis while *Sox9b* is expressed in the ovary (Chiang et al.,2001) [137]. These results have suggested that the chondrogenic expression of Sox9 is conserved among vertebrates, whereas the expression of *Sox9* in the gonad

seems to be somewhat diversified in fish species. According to Yokoi et al.,2002 [138] Sox9 is expressed in medaka in oocytes until the early vitellogenic phase in developing ovary, whereas the expression was not detected in developing testis. The presence of germ cells was confirmed by the vasa expression.

In the anuran *Rana rugosa*, two different typology of *Sox9* are found, *Sox9* ⍺and *Sox9 β*. *Sox9* ⍺ encodes a 482 amino acid protein containing the HMG box, whereas the other form *Sox9 β*, which completely lacks the HMG box. Sox9 expression was up-regulated in embryos after stage 16, and was seen in both developing testes and ovaries. In addition, *Sox9* ⍺expression was found prominently in the testis and brain among various tissues of adult frogs examined, and was considerably higher than *Sox9 β*. The fact that *Sox9* is expressed in both sexes suggests that this gene is involved in gonadal development of male and female frogs. This is dissimilar to the pattern in birds and mammals, in which *Sox9* expression is male-specific. It should be noted that the expression of *Sox9* ⍺and *β* was detected in the developing testis and ovary of the frog *R. rugosa*, suggesting that they are not a determinant for the testis development at least in this species (Takanase et al.2000) [139].

o) Whole mount in situ hybridization for the detection of mRNA .

Whole mount in situ hybridization (wmISH) is a highly sensitive procedure to detect the location of nucleic acids in situ, in their native location, and the resolution of details is unparalleled. Complex expression patterns in particular, can only be analyzed in whole embryos. The method has therefore by now been adapted to differen types of embryos and tissues as well, both from invertebrates and vertebrates and has become a general procedure in research laboratories studying questions of embryology and developmental biology.

In the original method DNA fragments were used that were labeled by random priming with DIG-dUTP. While this is still the method of choice if one wants to have a quick overview over the expression profile of a newly cloned gene, it has by now become clear that RNA probes can provide much better results (Lehmann R, Tautz D, 1994)[148]. Higher sensitivity can be obtained, because labeling efficiency is high and because they are single stranded. Moreover, since RNA-RNA hybrids are more stable than RNA-DNA hybrids, elevated hybridization temperatures can be employed, which result in a higher specificity and less background.

The detection of the hybridization signal is usually done with chromogenic substrates which develop a color at the place where the probe has bound. Different substrates are available, which allow to use different colors for differently labeled probes, e.g. biotin or haptens. Signal detection is realized with conjugated antibodies (alkaline phosphatase) against the respective haptene. Color reaction occurs by adding a colorless compound that becomes purple dye when phospatase is removed.

To understand the putative role of Sox9 expression during the male development we tried wmISH techniques in *B.bufo* specimen at different developmental stages in collaboration with Prof. Monica Bullejos Universitad de Jaen. Expression of Sox9 was detected using digoxigenin-labeled antisense riboprobes (which not include the HMG box) generated by PCR amplification of genomic DNA. Sox9 PCR product was cloned, linearized, DIG labeled and purified.

p) Whole mount in situ hybridization: protocol.

All steps were carried out in RNase-free conditions, using RNase-free solutions, RNase-free laboratories tubes and wearing gloves.

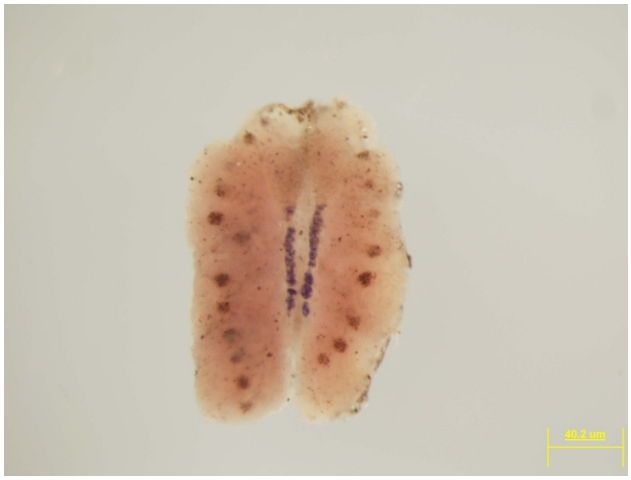
Tadpoles of *B.bufo* were previously anesthetized using with ethylene glycol monophenyl ether(Merk), dissected in PBS and fixed with 4% parafolmaldehyde (PFA) in PBS, pH 7,4 overnight at 4°C. Samples were washed twice with PBTX (PBS+0,1% Triton X-100), pH 7,4 for 10 minutes each at 4°C then dehydrated with MeOH/PBTX ascendant series (25%MeOH/PBTX→ 50% MeOH/PBTX→ 75% MeOH/PBTX → MeOH), for 10 minutes each. At this point, samples can be stored at -20°C for few months. Gonads were rehydrated by taking the back trough a MeOH/PBTX (75%MeOH → 50%MeOH→ 25%MeOH→ PBTX) series reverse then washed twice with PBTX for 10 minutes each. Samples were treated with 10µg/ml Proteinase K in PBTX for 20 minutes at room temperature, washed twice with PBTX for 5 minutes each, refixed with fresh 0.2% glutaraldehyde+4% PFA in PBTX (PBS + 0.1% Triton X-100) pH 7,4 for 20 minutes and rewashed twice with PBTX for 10 minutes each. Then gonads were placed in a 2ml eppendorf tube and filled with prehybridisation mix (50% formamide+ 5xSSC+ 2% Boehringer blocking powder dissolved directly into the mix+ 0.1% Triton X-100+ 0.5% CHAPS (Sigma)+ 1mg/ml yeast RNA (Sigma)+ 5mM EDTA+ 50µg/mL heparin; for hybridisation, add probe to 1 µg/ml solution 1) and incubated at 65°C overnight. After that prehybridization mix was removed, hybridization mix (including 1.0 µg/ml DIG labelled RNA probe) was added.

From this point on RNase-free conditions are no longer necessary.

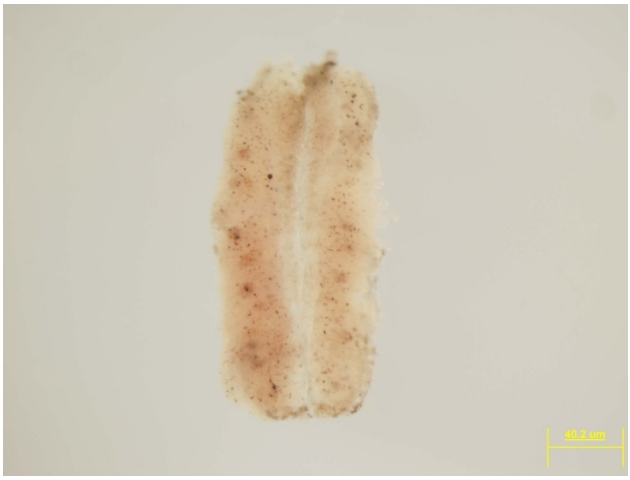
Samples were washed with the following solution for 5 minutes each at 65°C: 100% Solution 1 (50% formamide+ 5xSSC+ 0.1% Triton X-100+ 0.5% CHAPS), 75% Solution 1: 25% 2xSSC , 50% Solution 1 : 50% 2xSSC , 25% Solution 1 : 75% 2xSSC. During these washes, start preabsorbing the antibody by weighing out 3mg of embryo powder into a microtube, and adding 0.5ml of 10% sheep serum, 2% BSA in TBTX and 1µL anti-DIG-AP Fab fragment (Boehringer). After that, gonads were washed twice with 2xSSC+ 0.1% CHAPS and 0.2xSSC+ 0.1% CHAPS for 30 minutes each at 65°C, than twice with TBTX for 10 minutes each at room temperature. Gonads were breblocked with 10% sheep serum+ 2% BSA in TBTX for 2-3 hours at room temperature then replaced with the preabsorbed antibody. Samples were rocked overnight at 4°C, washed at least five times with TBTX containing 0.1% BSA for 1 hour each at room temperature (RT) and overnight at 4°C with TBTX containing 0.1% BSA. Samples were washed twice with TBTX for 15 minutes each, three times with NTMT for 10 minutes each and incubated with NTMT including 4.5µL NBT and 3.5µL BCIP (X-phosphate) per ml. At last, gonads were rocked for the first 20 minutes, transferred to a glass embryo dish and kept in the dark as much as possible to allow at the color reaction to proceed until signal is strongest without producing background staining. Reaction can be stopped by washing in NTMT(100mM NaCl, 100mM Tris.Cl (pH 9.5), 50mM MgCl2, 0.1% Tween-20) then TBTX overnight, then re-start the color reaction the next morning. When the color has developed to the desired extent, gonads were washed with NTMT then with PBTX, several times in PBS with 1% Triton X-100 then stains blue were fixed by incubating the gonads in 4% PFA in PBTX overnight at 4°C. Finally photograph gonads as soon as possible.

g) Results and discussion.

Stage IV13

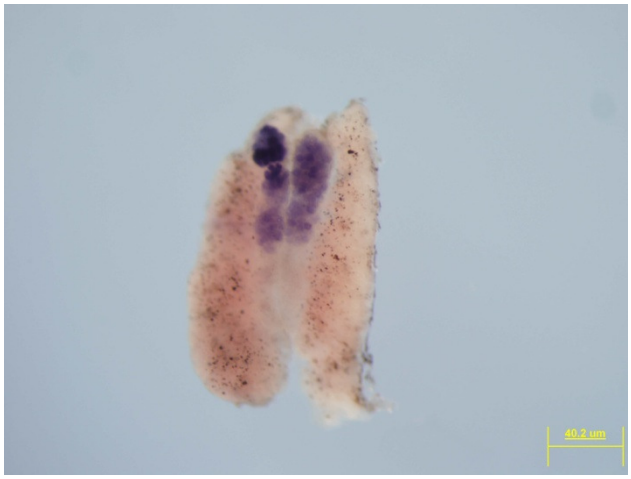


45 BbBO SOX9 n°1

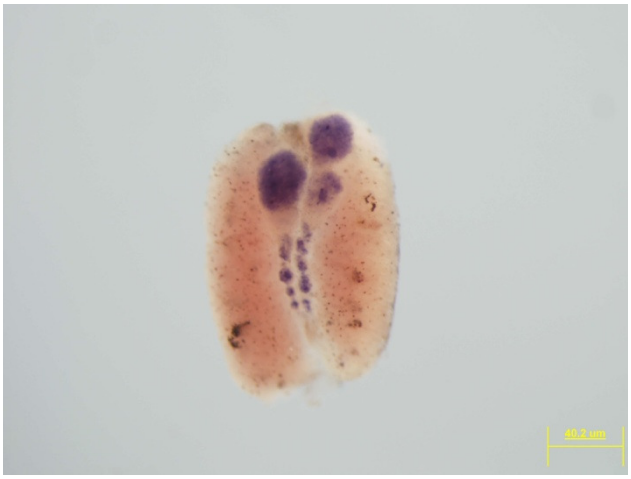


46 BbBO SOX9 n°68

Stage IV14



34 BbBO SOX9 n°72



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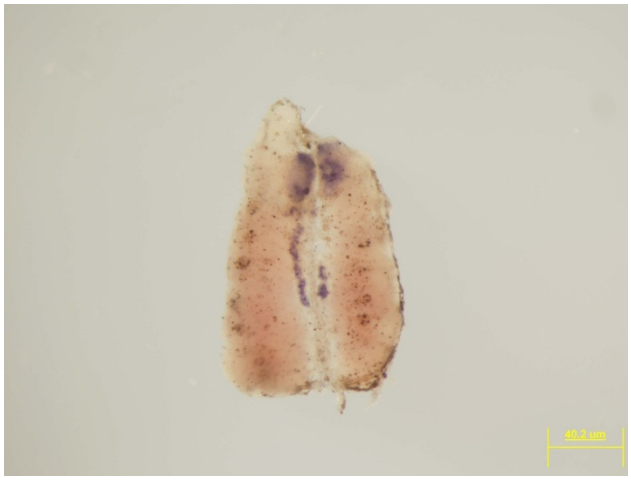
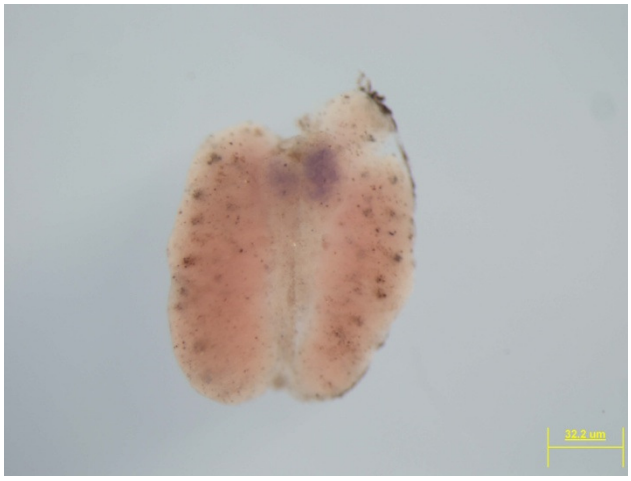


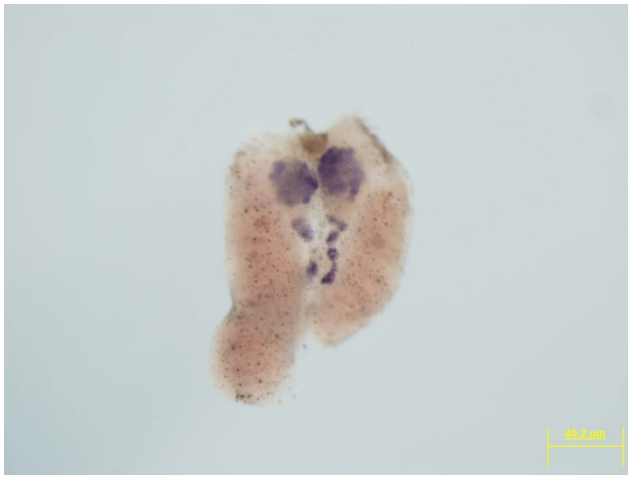
44 BbBO SOX9 n°13

Stage IV15



14 BbBO SOX9 n°4a

15 BbBO SOX9 n°4



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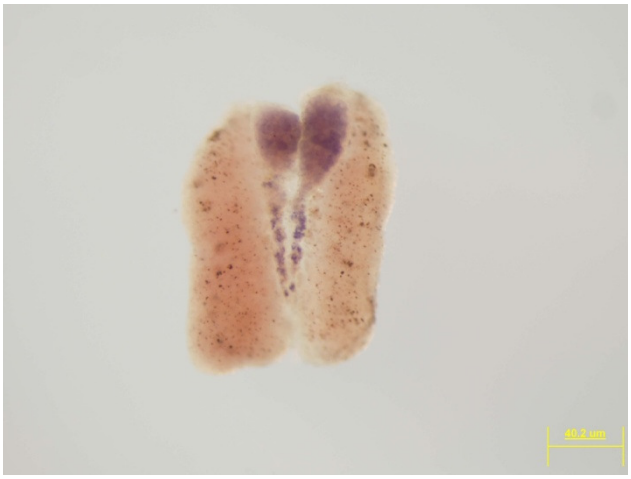

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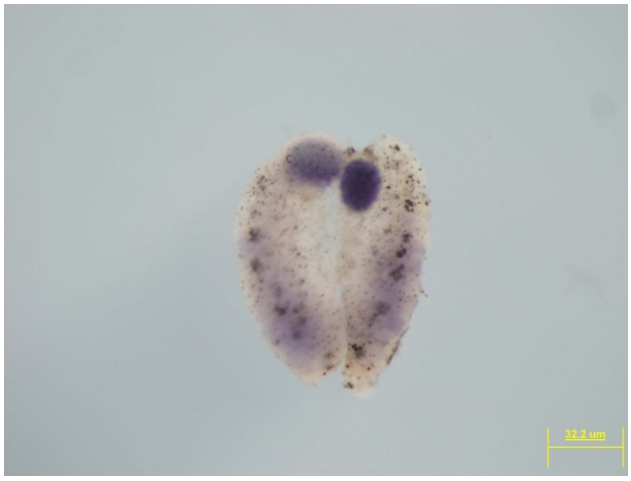
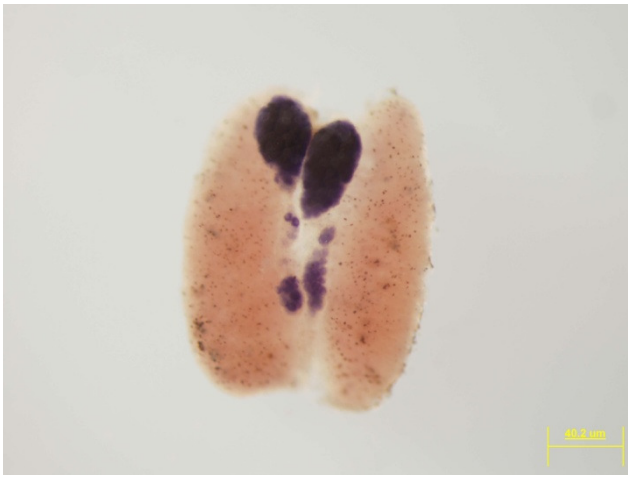
Stage IV16



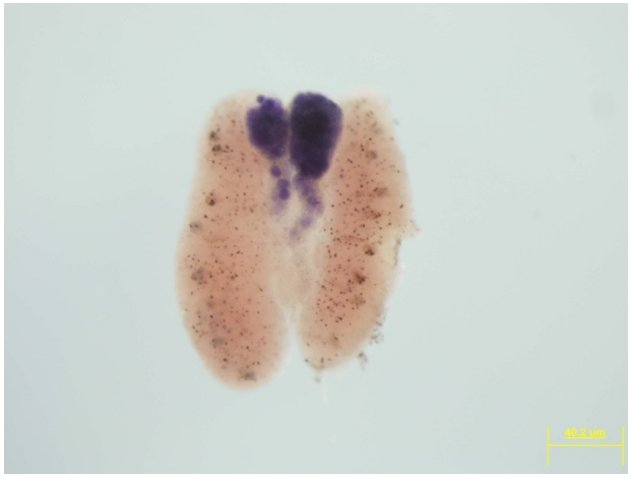
11 BbBO SOX9 n°27

12 BbBO SOX9 n°6

13 BbBO SOX9 n°11



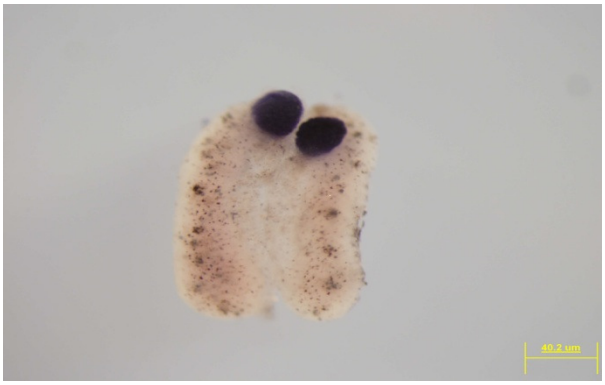
38 BbBO SOX9 n°77



39 BbBO SOX9 n°78

Stage IV 17

6 BbBO SOX9 n°31



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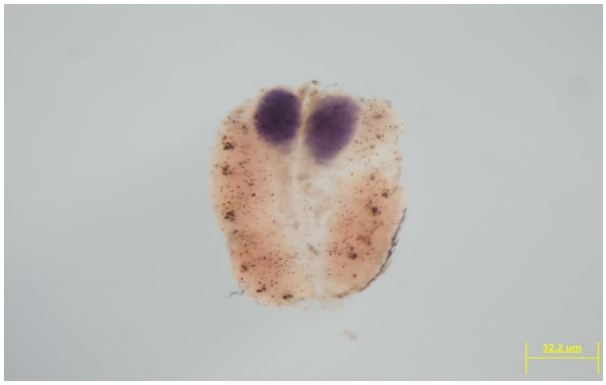

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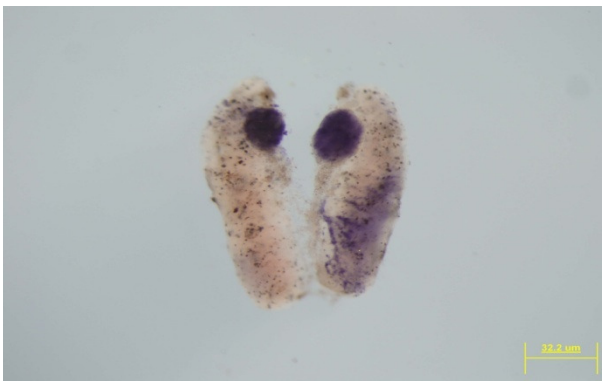

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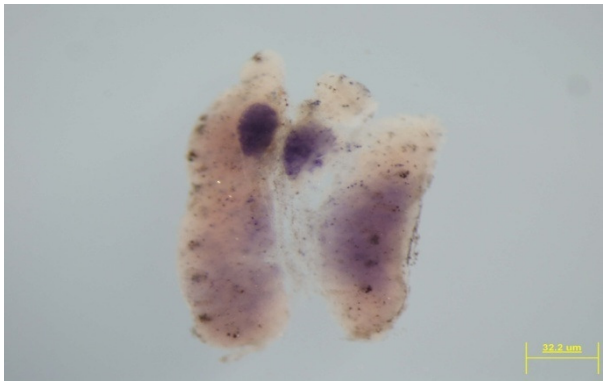
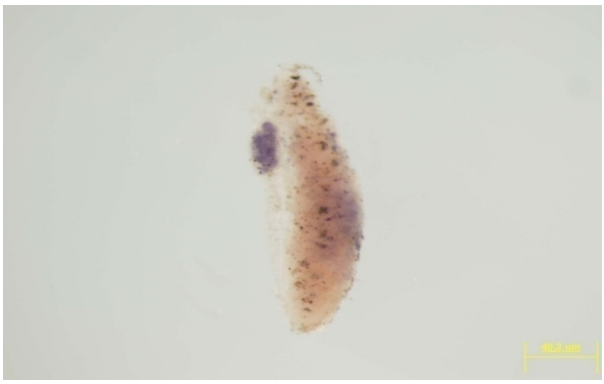


10 BbBO SOX9 n° 27



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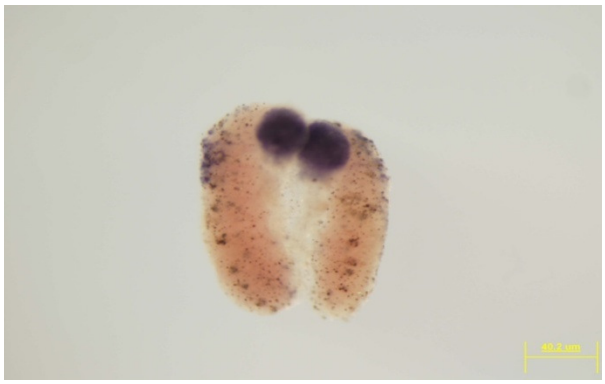

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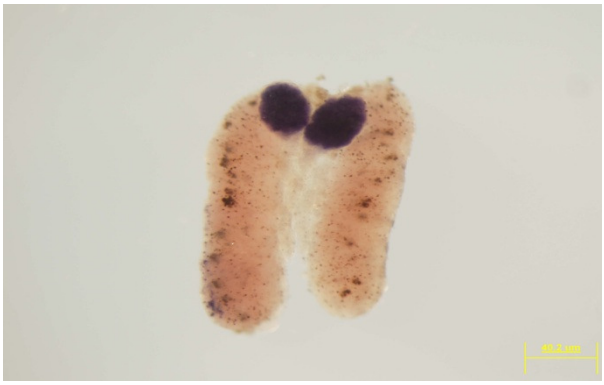
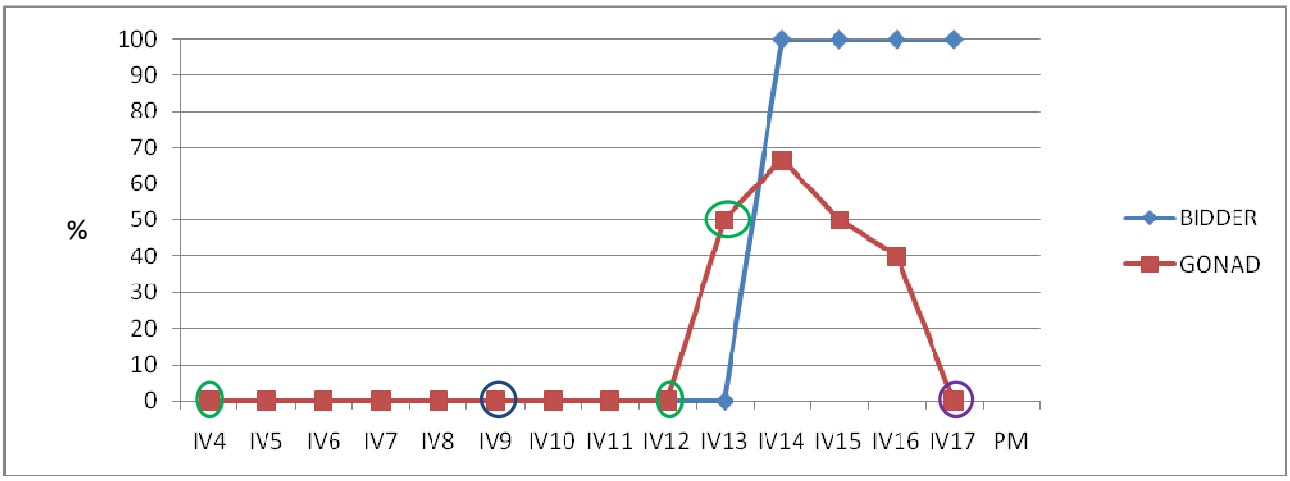


43 BbBO SOX9 n°36

Series 6: differential expression pattern during larval life (from stage IV 13 to stage IV 17) in *B.bufo* specimen. Stages from IV4 to IV12 are not shown. Scale bar 40,2 µm



Graph 3.2:Sox9 temporal expression during different developmental stages at gonadal level and in the Bidder's organ. This graph shows the presence/absence of Sox9 at the gonadal level (red line) and in the Bidder's organ (blue line) compared to the percentage of specimen examined. The colored circles represent the principal events in gonadal development.

Stage	Female gonad ♀	Male gonad ♂	Bidder's organ	Sox9 ♂	Sox9 B
IV4	undifferentiated	undifferentiated			
IV9	undifferentiated	undifferentiated	first ovogenetic wave		
IV12	ovarian cavity primordium				
IV13		centripetal migration of GCs inside the medulla		x	x
IV17			second ovogenetic wave		x

Table 3.9: principal morphological events in gonadal development. Table 1 resumed the principal morphological changes associated with the alternative development. x shows the positivity for Sox9 probe at gonadal and bidderian level.

Sox9 expression assayed by whole mount in situ hybridization (wmISH) with antisense riboprobes, has permitted to value the presence, the localization and the temporal expression in *B.bufo* specimen throughout the differential developmental pathways. Examining the experimental evidence we can define a general scheme:

- from stage IV4 until stage IV13: absence of the signal both in the Bidder's organ that in the gonad;
- stage IV 13: expression only at gonadal level;
- from stage IV14 until stage IV16: expression both in the Bidder's organ that in the gonad;
- stage IV17: expression only in Bidder's organ.

Gonad:

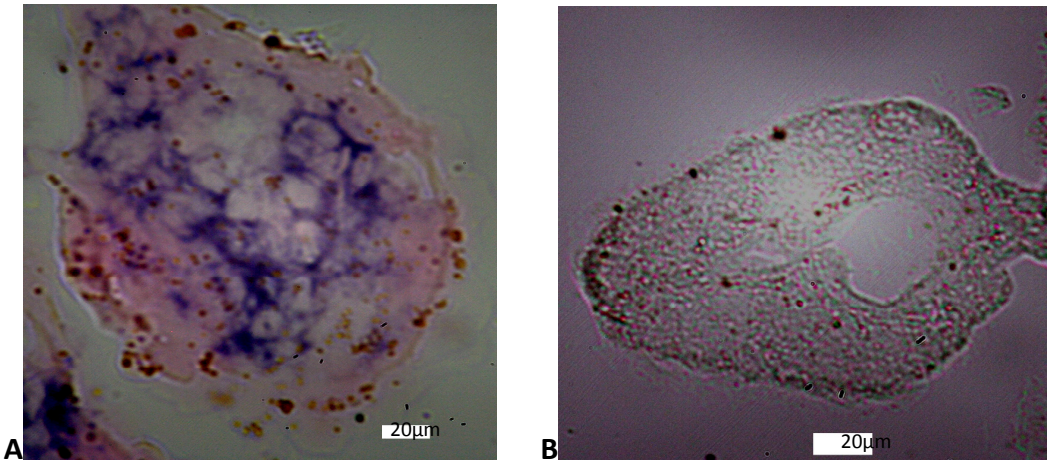


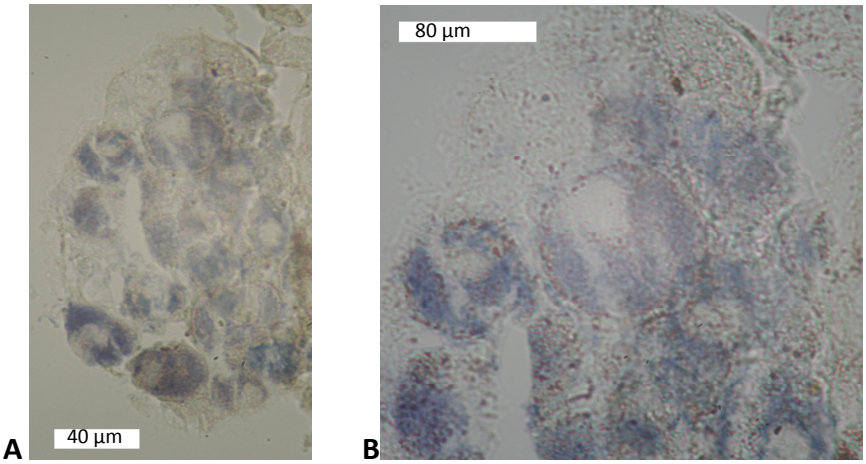
Figure 3.28: Sox9 expression in male stage IV16(A) and female stage IV16(B) gonads stained with eosin. Transversal sections. Scale bar 20 µm.

Sox9 expression at gonadal level is found in 50 % of samples during a range of developmental stages starting from IV13 (41° day) and ending to IV16 (44° day), with a temporal expression of 4 days. These results could give us some information about the choice of sexual differentiation pathway: only samples that express *Sox9* at gonadal level follow the male pathway whereas samples with no expression follow the female pathway. As reviewed for other vertebrate, also in *B.bufo Sox9* expression is detected in the medullar somatic cells of developing testis. Comparing the results of temporal expression underline in graph 2 with the principal events in gonadal morphogenesis highlight in table 3.9, we can argue:

- *Sox9* is expressied start from stage IV13 until stage IV17;
- at stage IV13 begin the centripetal migration of gem cells through the medulla;

From these evidence we could hypnotize a germ cell-soma during the male specific development. *Sox9* could represent a specific signal connected to morphological changes in male gonadal architecture

Bidder:



Figures 3.29: transversal section of Bidder's organ (stage IV14). Sox9 is present in the cytoplasm of the diplotenic oocytes. Scale bar 40 µm (A), 80 µm (B).

The presence of specific *Sox9* signal in Bidder's organ start from IV14 stage and remain certainly until the complement of metamorphosis (stageIV17) and maybe also in post metamorphic phase.
The probe is present at cytoplasmatic level in the 100% of samples either in males and females. The analysis referred to different samples highlight different expression patterns; *Sox9* signal is present in some dipoltenic oocytes of the first and of the second bidderian wave.
Considering that in mammalian *Sox9* acts not only during the testicular differentiation (in association with Sf1-steroidogenic factor 1 to promote the AMH gene transcription) but act also in other districts (*Sox9* gene expression occurs at sites of chondrogenesis). Different function of *Sox9* are present also in other vertebrates; it is reasonable that also in *B.bufo Sox9* could be related with more metabolic functions.

r) Spatiotemporal distribution of Sox9 in the gonad and in the Bidder's organ of B.bufo: discussion.

Data from our investigation support a differential role exerted by *Sox9* during testicular differentiation. This fact could be an additional evidence to support the asymmetric model. The interaction between germ cells and somatic cells will lead to the development of a male phenotype. Concerning the expression in the bidderian oocytes, the presence of Sox9 could be interpreted as a transitional phase involved in cellular processes not directly connected to the specification of the female gametes, but would therefore part of the information required for the cell cycle. Even in amphibians *Sox9* activity is not restricted only to the male gonadal somatic cells but would intervene in a series of processes; we can't consider only *Sox9* as a specific markers of testicular differentiation although it may give valuable indications on the differential pathway of differentiation.

4.DISCUSSION

The general issue of the research is to understand the germ-soma interaction during the alternative pathways of sexual differentiation.

Sexual determination and the subsequently sexual differentiation are two distinct but closely related mechanisms involved in the development of the typical sexual dimorphism associated to gonochoristic animals. Sexual determination is considered as a convergent adaptive phenomenon that takes place in accordance with a wide range of alternatives. Starting from an hermaphrodite information the alternative pathways are imposed by the conversion of the analogical signal into the binary switch initiating male or female development.

On the contrary, sexual differentiation is considered as a conservative phenomenon, quite similar in each classes of vertebrate, that leads to the development of testicular or ovarian architectures through pathways generally homologous. The bi-potential condition of the germinal cells trough oogenesis or spermatogenesis would be interpreted as a basic condition preceding the sexualizing action exerted by the somatic compartments or as a result of an inhibitory action exerted by the somatic cells on a primary developmental program.

In amphibians, the experimental results on sexual determination and sexual differentiation can be reconsidered by investigating the germ-soma interaction and the endocrine (paracrine) activity exerted by the somatic cells. Especially in bufonids, the natural condition of rudimentary hermaphroditism differentiation appears to be ideal for these kind of investigations.

The results of the different methodological approaches can be summarized:

a) Bio-molecular approaches on sex-determining mechanism:

- RAPDs primers have not provide the expected results; the limitation are related to the low percentage of detected polymorphisms and to the non reproducibility of the data. The reproducibility is a limitation associated with the technique: little difference in amount of DNA, annealing temperature and concentration of reagents can determine different bands patterns. For this reason we have to improve our protocol.

  Concerning the low percentage of polymorphism, the fact is partially due to a low number of tested primers (only 40). Increasing the number of primers more polymorphism are expected. Another problem related to the polymorphism is connected to the fact that polymorphic bands between males and females organisms could be associate only with phenotypic differences and not with sex-markers. Focusing the research on a couple of genomic DNA and by their offspring, we could increase the percentage of sex linked polymorphisms.
- Concerning the AAT gene, no difference in alleles sequences are identified in the homogametic ZZ and heterogametic ZW sex as reviewed for other amphibian (*Xenopus, R.sylvatica*). For this reason, AAT sequence is not useful to discriminate the sex of the samples and it is not useful to the purpose of our investigations.

b) Sex- reversal in *B.bufo* specimen.

- Searching missing pieces in developmental mechanism by using sexual reversal have not produce the expected results.

  Hormonal treatments have underline different evidence. The mortality rate seems to be the limiting variable; specimens treated with hormones shows a higher mortality if compared to control (table 3.4): hormonal treatments seems to exert a toxic action. Comparing the resulting sex ratio in specimen treated with estradiol, data underline a difference statistically significant trough a female development. Moreover, while hormonal treatments with testosterone seems to be toxic for male and female specimen, estradiol seems to act in a differential manner resulting toxic only on males specimen; during their embryonic life, genotypic female tadpoles survive whereas genotypic male tadpoles die before reaching the stage IV17. Exogenous dosages of estradiol could alter the male developmental pathway increasing the mortality rate in male specimen. However, hormonal treatments seems not induce a unique sexual phenotype, and this poses serious limitations for the purposes of research. A hypothesis to test is to anticipate or prolong the exposure at estradiol increasing the number of organisms, or to rear tadpoles in presence of estradiol at low temperatures (because low temperatures seem to act as epigenetic factor inducing a feminizing effect on the larvae in development).

- Concerning sexual reversion by castration, the incapability to produce an offspring by the mating between “normal” and “inverted” males have not give us information about the chromosomal sex determining mechanism. For this reason it was not possible to investigate the gonadal development of a unisexual offspring, and so it was not possible to study the interaction between somatic and germinal districts during male development. The causes of death are unknown. Treatments with hormones can reduce the normal life by producing tumors in various districts (with a dose dependent trend), but the comparison between inverted and normal males showed a quite similar rate of body growth and a longevity. Probably the limiting condition is related to the captivity. We can confirm that orchiectomy induce a sexual reversion and that organism treated with DES accelerate the bidderian oogenesis. By dissecting the specimen, we can confirm the results obtained by Zaccanti and Tognato (1976) [114]. Using a dosage of 1ug DES/week the macroscopic examination of the Bidder’s organ two years after orchietomy show a greater increase in volume, and a great number of the usually abortive large diplotenic oocyte initiate a massive vitellogenesis reaching the “third growth period”. In orchiectomized males, the Bidder’s organ appear much greater in volume than those of intact control males but oocytes don’t reach the vitellogenetic phase.

c) Sexual differentiation.

- The evidence obtained from the investigation of the alternative gonadal morphology by detecting the distribution of α-tubulin by confocal laser scanning microscopy can be synthetically summarized: the Bidder's architecture is quite similar in males and females as confirm of the ovarian character of the organ itself; the organogenesis of the ovary in genetic females appear as a conservative pathways that maintains substantially the previous undifferentiated architecture with the germinal cells located in the peripheral compartment and the somatic cells in the centre of the gonad whereas the organogenesis of the testicle seems to happen as a kind of sexual inversion with germinal cells located in the center of the gonad as a consequence of a centripetal migration.

  No difference in shape are detected in the germinal lineage of males and females gonads; the sexuality can be determined by only morphological differences of cellular compartments: peripheral in females (as in undifferentiated samples), central in males.
- The results obtained from the investigation of the specific localization of the germinal cells by detecting the positivity for *Vasa* by confocal scanning microscopy can be summarized: *Vasa* protein is detected only in the cytoplasm of germinal cells of each gonadal district (Bidder or gonads). The Bidder's organ present a single pattern equal in males and females and the associated signal decrease during oocyte differentiation, higher in gonial cells or synaptic oocytes and lower in diplotenic oocytes.

  The female architecture present a bipartite architecture with the germinal cells located in the peripheral compartment and the somatic cells in the centre of the gonad whereas the male architecture present germinal cells in the center of the gonad as a consequence of a centripetal migration.

  No difference in shape are detected in the germinal lineage of males and females gonads; the sexuality can be determined by only morphological differences of cellular compartments.
- Concerning *Sox9* differential expression. The pre –Sertoli cells of the genital ridge are the only cells whose require the presence of the Y chromosome in order to induce the bi-potential gonad to develop the testis: based on this evidence, it is consider that SRY express its role in these cells. This means that the pre-Setoli cells are able to direct other cellular district in order to develop following a male phenotype. One of the intercellular target gene induce by *Sry* is *Sox9*. The indirect action of *Sry* would be mediated by *Sox3* and *Sox9* genes. The gene involved in the activation of the testicular organogenesis would be *Sox*9: in females SOX3 protein could inhibit the action of *Sox9* leading to the development of the default ovarian pathway. The actual mechanisms of sexual differentiation in mammals would be the result of an ancient mechanism based on the dosage of *Sox3*, by which would be evolved *Sry*. The developmental control of male or females is a system strongly canalized: once the decision to begin the development of the testis is taken, all the cells of the primordial gonad are 'recruited', with a finely controlled mechanism.

  From the analysis of differential expression patterns of *Sox9* in *B.bufo* we can affirm that *Sox9* expression is present in 50 % of samples during a range of developmental stages

starting from stage IV13 (41° day) and ending to stage IV16 (44° day), with a temporal expression of 4 days. Comparing the results of temporal expression with the classical morphogenesis of gonadal development, we can underline that the presence of *Sox9* in somatic cells, starting in IV13, could be relate whit morphological changes. From these evidence we can support a significant role played by *Sox9* during the male specific development. *Sox9* could represent a specific signal connected to morphological changes in male gonadal architecture. Considering the presence of specific *Sox9* signal in Bidder's organ, it start from IV14 stage and remain certainly until the complement of metamorphosis (stageIV17) and maybe also in post metamorphic phase.

In the Bidder's organ the probe is been detected at cytoplasmatic level of oocytes of 100% of samples either in males and females. The analysis referred to different samples highlight different expression patterns; Sox9 is not present in stage IV9 (first ovogenetic vawe) but the signal is present in some dipoltenic oocytes of the first and of the second bidderian wave at the complement of metamorphosis (stage IV17).

Considering that in mammalian *Sox9* acts not only during the testicular differentiation (in association with Sf1-steroidogenic factor 1 to promote the AMH gene transcription) but act also in other districts (*Sox9* gene expression occurs at sites of chondrogenesis for a correct development), and also act in different tissues in other vertebrates, is reasonable that also in *B.bufo* Sox9 could be related with more metabolic functions. For these reasons, the positivity in Bidder' organ, could be interpreted as a transitional phase involved in cellular processes are not directly connected to the specification and the differentiation of the female gametes, but would therefore part of the information required for the normal cell cycle. Even in amphibians *Sox9* activity is not restricted only to the male gonadal somatic cells but would intervene in a series of processes; we can't consider *Sox9* as a specific markers of testicular differentiation although it may give valuable indications on the differential pathway of differentiation.

Taken together, data collected from the investigation of the alternative sexual differentiation by different methodological techniques, support the asymmetric model. Germ cells have an innate tendency to develop, without a somatic input, through a female phenotype. This statement is confirmed by data obtained by confocal laser scanning microscopy investigations on the alternative gonadal morphology by detecting detection of α-tub. Female gonads present a bipartite architecture that resemble an undifferentiated condition with germ cells in the cortex and medullar cells reducing as a mono layers of somatic cells delimiting the ovarian cavity. The bi-potent condition would be the result of a somatic inhibitory signal on a primary ovarian sexual pathway. Male gonads present an alternative morphology with the germ cells located inside the medulla. Data are confirmed by the analysis of the distribution of germinal cells by detecting *Vasa* protein. In addiction the whole mount in situ hybridization have underline differential expression patterns at gonadal level. Considering that *Sox 9* expression appear in coincidence with the begin of the centripetal migration of germ cell inside the medulla we can argue that the germ cells-soma interaction in male gonad lead to the male phenotype as result of an inhibitory action on this general scheme.

The evidences that the anuran share a general mechanism of sexual differentiation similar with other vertebrates, consolidates the putative conservative role of sexual differentiation throughout the evolutional pathway whereas sexual determination would be an adaptive mechanism (Zaccanti,1992) [143].

Research means having clear objectives and uncertainty results that, as often happens are unexpected and stochastic. Searching for new experimental evidence to understand the dynamics of differentiation and determination of sex, I have probably extended those who are now gaps in knowledge, doubts and uncertainties.

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