Journal of Molecular Histology & Medical Physiology

Exbrayat et al., Mol Histol Med Physiol 2019, 4:1

Review Article Open Access

Amphibians as Models for the Study of Cell Proliferation, Differentiation and Apoptosis throughout Embryonic Development and Oviduct Cycles

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Received date: February 20, 2019; **Accepted date:** March 04, 2019; **Published date:** March 08, 2019

Abstract

Amphibians are anamniotic vertebrates having conquered the terrestrial environment. Their reproduction and development occur in water. After metamorphosis, the animal leaves the water to live on the ground. Yet, some species remain in the water and certain of them are viviparous. Since a long time, amphibians are used such as animal models to understand physiological or developmental mechanisms. Today, amphibians can be used such as excellent models to understand the importance of cell proliferation, differentiation and death throughout embryonic development. A second way concerns the importance of these phenomena in the organs subjected to very sharp variations, alternation of differentiation/dedifferentiation throughout the sexual cycle, according to external or experimental conditions. The importance of apoptosis and of the presence of calpains has been noted throughout embryonic development of *Xenopus laevis*. In several caecilians, the spectacular variations of oviducts throughout the sexual cycle are linked to seasonal alternations, and under the control of several hormones and their receptors.

In this review, an overview of works performed by our laboratory is given. The models used were the anuran *Xenopus laevis* in order to study the apoptosis from fertilization to metamorphosis and several caecilian amphibian species in order to understand the interactions of these phenomena during the alternation of differentiation/dedifferentiation of the oviducts throughout the sexual cycle.

Keywords: Amphibian; Development; Reproduction; Genital tract; Oviduct; Apoptosis; Cell death, Cell proliferation; Cell differentiation

Introduction

Phylogenetically placed between fish and reptiles, the amphibians are anamniotic vertebrates having conquered the terrestrial environment [1]. The Amphibian class is divided into three orders: Anurans group frogs, toads, tree-frogs, Urodelans group newts, salamanders and other curious forms such as the amphiuma, siren or olms, and Caecilians (or Gymnophionans), a very homogeneous group of lengthened burrowing or aquatic species, without belt nor legs, living in tropical or equatorial areas. Amphibians are found throughout the earth, in temperate as well as tropical areas, even in the oases in arid areas.

The reproduction and development of all the amphibians occur in water. After metamorphosis, the young animal leaves the water to live on the ground; generally, it stays close to a wet biotope or it buries itself in the substrate [1]. Nevertheless, some species remain aquatic or return to the aquatic life in the adult state, and some of them remain terrestrial. They can also present very specialized adaptive mechanisms regarding reproductive patterns, from oviparity to marsupials [2]. Since a long time, amphibians have attracted the interest of researchers. Aristotle (384-322 BC) was probably the first to report the metamorphosis of frogs [3,4]. Later, a lot of anatomical and biological aspects comprising reproduction and development of amphibians were studied by zoologists. Throughout the XIXth century, frogs and toads were used such as animal models to understand physiological mechanisms. Amphibians were also used as models to understand the main steps of embryonic development [5]. At the end of XIXth, frogs, toads, and newts allowed the searchers to understand the main principles of embryonic development [6] and more recently, they were used to understand the importance of the apoptosis (Programmed Cell Death or PCD) during the developmental stages [5]. Today, the interests in studying amphibian species remain multiple. Some species living in hard-to-reach areas are certainly still unknown with perhaps unique adaptations: some very peculiar modes of reproduction have been described [2], and it might be possible to find new modes of adaptation. It is also possible that certain species modify their living mode according to climate change. Living first in an aquatic environment, then in a terrestrial one, after a metamorphosis during which the animal is particularly fragile and sensitive to the environment, the amphibians are also used such as bio-indicators in order to understand the quality of water or to appreciate the toxic effects of chemicals [7]. The skin of these animals develops several efficacious antibiotics or opioid molecules of interest in the pharmaceutical industry [8,9].

In order to understand the importance of cell proliferation, differentiation, and death, amphibians can be used such as excellent models in two ways. The first way is by considering embryonic development mainly documented in species for which a normal development table has been established. In the majority of cases, the amphibian being oviparous, their development occurs in water and it is easy to survey it in a Petri dish [10]. These animals have therefore been well used in order to understand developmental mechanisms. The second way to use amphibians as models is by studying relevant organs variations under natural or experimental conditions.

This review is aimed to give an overview of studies-mainly performed in our laboratory-using several amphibian species such as models to understand the importance of cell proliferation, differentiation, and death. The models used to study the importance and interactions of these phenomena were the anuran *Xenopus laevis* during embryonic development, and several caecilian amphibians during the alternation of differentiation/dedifferentiation of the oviducts throughout the sexual cycle.

The Interest of Amphibians to Study Proliferation Apoptosis and Differentiation Throughout the Development

Since several years, Amphibians are used to understand the importance of apoptosis in development. Periodically, it is useful to give a state of the art of this importance. Several reviews of this point were already published [5,11,12]. Among amphibians, *Xenopus laevis* was particularly used to study programmed cell death [12-15]. Some other species have also been used for the same purpose.

General aspects of amphibian development

After fertilization, eggs immediately divide into several blastomeres during the phase of cleavage at which the size and the shape of embryo remain the same. At the end of cleavage, an inner cavity, the blastocoel, is observed between the cells. The maternal mRNAs previously accumulated in the oocytes are distributed into the cytoplasm of blastomeres. Before a physiological step, the Mid Blastula Transition (MBT), these maternal mRNAs encode for proteins which trigger the expression of zygotic genes. At this time, the zygotic genes do not express except those implicated in membrane building. In post-MBT, zygotic genes express according to maternal signals. After cleavage, at gastrulation, cells are organized in several layers: ectoderm, mesoderm, and endoderm. The archenteron or primitive intestine is observed. After gastrulation, on the dorsal part of the embryo, the neural tube forms. The notochord and somites differentiate from mesoderm. After neurulation, organogenesis occurs followed with a growth period, at the end of which metamorphosis occurs. This phase corresponds to the transfer from an aquatic to terrestrial life and is characterized by the modification, development, or regression of several organs.

Distribution of apoptotic cells throughout amphibian development

Apoptosis occurs quickly in development. A first program under the control of maternal genes is observed before MBT in order to eliminate damaged cells, and a second program occurs at the onset of gastrulation. Apoptotic cells are observed during neurulation, organogenesis and they are abundant at metamorphosis. In *X. Laevis*, apoptotic cells begin to appear at the onset of gastrulation on ectoderm and internal mesoderm. At the end of gastrulation, apoptotic cells are distributed according to one or two dorsal median strips, showing the place of future neural plate [16]. In the newt *Cynops pyrrhogaster*, dead cells display the same repartition [17].

Some experiments showed that embryos continued to cleave after the eggs were submitted to treatment damaging DNA, showing that embryonic DNA was not implicated in the control of cell cycles. In two cells-embryos submitted to treatments affecting transcription, segmentation was not affected. In embryos submitted to treatment affecting protein synthesis or DNA replication, the cleavage was stopped. A lot of cells became apoptotic and embryos died at the onset of gastrulation. Caspase activity was found in extracts of irradiated embryos at stage 10.5 [18-20]. The first program of PCD was activated at the onset of gastrulation, consequent to the degradation of cells occurring before MBT. The second program of PCD started at gastrulation in normal embryos. At the onset of gastrulation, the zygotic transcription was necessary to suppress apoptosis in normal embryos [21]. The maternal program was blocked using an inhibitor of apoptosis such as Bcl-2 [22]. At MBT, the maternal program can start apoptosis; but this program can be blocked by zygotic transcription [22]. In *X. laevis*, the maternal program was activated just after MBT with an over-expression of S-adenosyl methionine carboxylase which is a crucial actor of the methionine cycle known to feed the methylation activities in Vertebrates [23-25]. Indeed, several experiments have shown some molecules involved in methylation processes have an impact on the starting of apoptosis. In embryos injected with 5-aza-CdR inducting hypomethylation of DNA, or 5-methyl dCTA, inducting contrarily hypermethylation, the cleavage was normal but the embryos became apoptotic at gastrulation [26,27]. Apoptosis was suppressed in embryos injected with an inhibitor of caspases [27], and apoptosis was observed at gastrulation in two blastomeres embryos in which SAMDL mRNAs were injected [28,29]. Other molecules such as MBD4 and MLH1 have been signaled to participate in the checkpoint of apoptosis [30]. The anti-apoptotic factor p27BBP/eIF6 was related to an increase of apoptosis in the parts of embryos needing cell death for harmonious development [31]. Several genes have been shown to be crucial for tissue differentiation but not for gastrulation, but their overexpression induces damages in DNA [32]. Moreover, an over-expression or a depletion of Bix3 causes apoptosis [33]. At last, maternal p53 mRNAs seem to be essential for *X. laevis* development [34].

At neurulation, the presence of apoptotic cells was detected in *X. laevis* and *C. pyrrhogaster* [16,17]. During neurulation, the apoptotic cells first underlined the neural plate, the neural fold, the neural tube. When the brain was first observed, a lot of apoptotic cells were detected at the level of primary sensorial neurons in olfactive and otic placodes, and also in the notochord [35]. The first wave of apoptosis affects neurons prior to the formation of synapses. The territories of retina result from the repression of Xotx2 by Xrx1. Xrx1 is involved in the development of the anterior brain and eyes [36].

In *X. laevis*, several experiments showed that PCD regulates primary neurogenesis at the level of neuronal determination [37]. Several genes such as POSH (Plenty of SH3s), XBtg2, msx1, Slug, Xphb1 are involved in the development of the anterior brain, comprising PCD and proliferation of neurons [38,39]. The effects of genes can be antagonist: the formation of neural crest cells is controlled by the balance between Slug and msx1 antagonist genes [40,41]. Both the anti-apoptotic gene Bcl-2 and the apoptosis-promoting gene Bax disturbed the expression of several other genes. Prohibitin 1, following the expression of Xphb1 gene, displays function in the development of neural crests [42-44]. E2F1-binding domain is necessary for the action of Xphb1 in the development of neural crests [45]. Several genes and proteins are involved in the decrease of proliferation: p27BBP/eIF6 [46,47].

During organogenesis and growth period, apoptotic cells were observed in the whole organism but during the aquatic phase, the specific organs are not affected by apoptosis. In the nervous system, some apoptotic cells were observed between stage 35 and 39 in different parts of the eyes [47]. Then, approaching metamorphosis,

when hind limbs begin to appear, the number of apoptotic cells increases first in the epidermis, and then in the second generation of gills, the cement gland, affecting finally the nervous system and several organs which display a large number of dead cells. At these stages, the tadpoles become very fragile and many of them die [12,48].

Amphibian metamorphosis involves a strong remodeling of organs. Larval organs are replaced with adult ones, and a wave of apoptosis occurs. The pattern of apoptosis in amphibians has been already well reported at metamorphosis stage [5]. During this period, apoptosis and cell proliferation have both been observed in all the tissues. Nervous tissues have been well studied [49-51]. It was supposed that the process of optic nerve remodeling accompanied the displacement of eyes from lateral to a more dorsal and rostral position as the frog acquired binocular vision [52-56]. Apoptosis also affects muscle structure at metamorphosis [57-61]. In the larval intestine, a lot of cells have become apoptotic and were replaced with non-differentiated cells in order to form the adult intestine. Most studies in several amphibian species document the anatomical, histological and molecular aspects of the passage from a larval digestive tract to an adult one [62-65], and more particularly the intestine [66-69]. The outer cell layers of the epidermis die by apoptosis during the climax [70]. At metamorphosis, numerous larval red blood cells also decrease, whereas adult ones increase. Many larval red blood cells expressed TUNEL-positive reactions [71].

Effects of calpains on the embryonic development of amphibians

Several studies indicated the importance of calpains in the apoptosis of cells during embryonic development of *X. laevis* used such as a model. Calpains are implicated in cell differentiation, proliferation, migration and apoptosis [72], more especially throughout the development [73-76]. Calpain family groups proteases found in all vertebrates [77]. More especially, the amphibians possess a single gene encoding for CAPN 1, 2, 8, 11 and several specific genes encoding for CAPN 3, 9, 12, 13 and 17 [78,79]. In *X. laevis* it has been shown that calpains are involved in the different phases of embryonic development: gastrulation and neurulation [80], organogenesis and metamorphosis [5,81]. They play an important role in apoptosis occurring during embryonic development [82,83]. The expression of calpains was researched throughout the development of *X. laevis*, using real-time RT-PCR and immunohistochemistry. During the early stages, the calpains expression was weak. XCl-2 was the first calpain to be initiated at the gastrula stage and reached a maximum at neurula and tailbud stage. Expression of calpain 1 isoforms was mostly restricted to the tailbud stage. The amount of calpain 3 was particularly high during the organogenesis and growth. But calpain 3 mRNAs were lower than that of calpain 1 and calpain 2. Calpain 3 was the only enzyme strongly expressed at metamorphosis. These calpains are involved in organogenesis. These results are concordant with the results of western-blotting and immunohistochemistry [22,23,80,83].

The apoptosis can be regulated by changes in calcium rate. These changes are linked to the activity of several receptors such as ionotropic glutamate receptors [84,85]. In order to know the relationships which could exist between the calpains and apoptosis throughout the development the expression of these proteases was researched on eggs, tadpoles, and adult tissues of individuals in contact with several drugs acting on glutamate receptors [82]. The presence of calpains was researched on skin and gills, intestine, dorsal muscle, heart and brain [82] in *X. laevis* embryos and tadpoles submitted to different concentrations of different drugs acting on the glutamate or kainate receptors [82]. The results showed that in skin and gills, a three-hours exposure to 30-120 mM Glu resulted in a concentration-dependent increase of apoptosis. In internal brain, heart, intestine and muscle, the effect was not progressive and a dramatic increase in apoptotic activity after exposure with 120 mM Glu was observed. The largest number of apoptotic cells was noticed in the skin and the gills. At an exposition to 0.015-0.75 mM KA, PCDs were observed in all tissues. The number of "Apostain"-stained cells was 9 to 34 times larger than the control in the skin, the gills, the intestine, or the dorsal muscle, according to the organ. The largest number of apoptotic cells was noticed in the skin and the gills.

All these cases from recent research illustrate that amphibians, and more especially *X. laevis* are useful models to study the regulation of embryonic development.

The Interest of Amphibians to Study Proliferation Apoptosis and Differentiation Throughout the Cyclic Variations of Genital Tracts

Needing an aquatic environment for reproduction, amphibians perform a lot of reproductive patterns narrowly linked to seasonal alternations [1,86-89]. In Anurans, Urodelans or Caecilians, several species perform continuous cycles in both males and females. In these species, the genital tracts are always ready to start the reproduction process. These animals are usually found in areas where the rainy seasons do not return regularly from one year to the next, so they are ready to breed at once rain occurs. In these species, testes always contain all the stages of spermatogenesis and ovaries contain vitellogenic oocytes ready to be laid. In females, the physiological structure of oviduct is always the same with a flexuous anterior part and another part in which the envelopes of oocytes are deposited [90].

In the three Orders, several species perform discontinuous cycles, in males as well as in females [1,86-88]. In this particular case, the testes can present a period at which spermatogenesis occurs contributing to filling testes which are then ready for breeding. After breeding, testes remain empty with only spermatogonia until the next period of reproduction. In other species, after breeding, a stock of spermatozoa is reconstituted in order to be used at the next period of breeding which is often distant of several months. In females, vitellogenic oocytes are evacuated at ovulation, then ovaries contain only non-vitellogenic oocytes, and vitellogenesis occurs before the period of breeding. During the period of quiescence, the lumen of the oviduct is bordered with undifferentiated cells with a high nucleocytoplasmic ratio. When the preparation of breeding occurs, the epithelium differentiates in several cell types surrounded with a connective tissue also differentiated. In oviparous species, the oviduct finishes being differentiated into several parts: pars convoluta, pars recta and pars utera. These parts are bordered with gland cells which elaborate the different layers of the envelope surrounding the future egg. In oviparous Anurans and Urodelans, fertilization is external. Yet, it can be internal in some Anurans (*Ascaphus*), Salamanders and newts with the use of a spermatophore disposed on the ground by the male, which is intromitted into the female cloaca [91]. In Caecilians, fertilization is always internal, and the animal can be oviparous, viviparous, or direct-developing [91]. In all the viviparous species, the oviduct is differentiated in several parts, the first one is the part where fertilization occurs, the posterior part being the uterine part in which development occurs [86,89]. During these cycles, the oviducts are the seat of several fundamental phenomena: cell differentiation,

proliferation, and death. This makes the oviduct a good candidate to look for epigenetic variations involved in differentiation and morphologic modifications in response to environmental (seasonal) change.

Differentiation of the oviducts

In all Amphibians, the female genital ducts begin with a ciliated funnel opened into the general cavity. After ovulation, the oocytes attempt the funnel after a more or less long progression into the general cavity. In Anurans and Urodelans, the wall cells produce some secretions which surround the oocyte. The nature of those secretions is variable throughout the oviduct.

Among the Amphibians, several Caecilians species have been more particularly investigated in order to understand the variations of oviducts throughout the reproductive cycle [92-94].

Boulengerula taitanus is a direct-developing caecilian living in Taita Hills (Kenya, Africa). It is characterized by a phase of quiescence from March to August, followed with a phase of preparation to reproduction in September and a phase of breeding from November to February with 5-10 laid eggs [95]. Some morphological changes of the epithelium bordering the lumen of the oviduct and the connective tissue are observed throughout the year. Between November and February, the oviduct exhibits the highest degree of development of secretory cells. In the pars recta, the lumen is bordered with a columnar epithelium composed of ciliated cells. Between the villi forming some crypts, gland cells present a cytoplasm with tiny granulations of sulfate and/or carboxylic acidic proteoglycans. The secretion is certainly of the holocrine or apocrine type. In the pars convoluta, the wall of the lumen has developed villi or rounded fingerings. Its wall is bordered with ciliated cells intercalated with small goblet cells filled with sulfate and carboxylic acidic proteoglycans. Some gland cells situated in the crypts between the villi possess an abundant often granular cytoplasm with blue-stained with azan trichrome and PAS-positive secretions. The connective tissue is thick, with wide blood vessels just under the crests of the villi. The lumen of pars utera is bordered with flattened high crests covered with ciliated cells. In the chorion, crypts contain glands with a single cell type. The staining of the secretions of these cells showed the presence of both sulfate and carboxylic acidic secretions. Wide blood vessels are also observed in connective tissue. In the pars recta, the lumen is bordered with a columnar epithelium composed of ciliated cells. Between the villi forming some crypts, gland cells present a cytoplasm with tiny granulations of sulfate and/or carboxylic acidic proteoglycans. The secretion is certainly of the holocrine or apocrine type. Between March and August, the oviduct is poorly developed. Whatever the part, the epithelium bordering the narrow lumen has regressed. The nucleus of several cell types appeared pyknotic. The lamina propria is narrow and contains few blood vessels. In September-October, the oviduct develops, the diameter of the lumen increases and the epithelium becomes thickest. All the secretory cells increase in size and are filled with secretions.

Typhlonectes compressicauda is an aquatic viviparous species living in South America. A population coming from French Guyana has been more particularly studied [96]. The females of these species present a biennial sexual cycle. At the beginning of the first year of the cycle, in October (middle of the dry season), the ovaries presented more and more vitellogenic oocytes. At the beginning of the rainy season in December, the females are ready to breed. Ovulation occurs in February. After fertilization, the development of embryos occurs entirely into the uterus of females during 6-7 months. Parturition takes place in September-October (dry season); 6-8 newborns can be observed living near their mother into the mud. At this time, the ovaries no longer contain any vitellogenic oocytes. The second year begins with a new vitellogenesis, but next February, the ovulation does not occur. Vitellogenic follicles degenerate and become atretic, the ovaries decrease and remain inactive until the following October, during which time a new process of vitellogenesis occurs [94].

The oviducts are submitted to seasonal variations. At ovulation, the oocytes attain the tubal part of the oviduct. During sexual quiescence, the wall surrounding the funnel or the lumen is bordered with a single layer of undifferentiated cells, with a high nucleocytoplasmic ratio.

Just before ovulation, the connective tissue increases, and the different parts of the oviduct differentiate. In the funnel, the epithelial cells ciliate. At ovulation, some crests have developed limiting crypts containing cells with protein secretions. After ovulation, the funnel regresses. At the second year of the cycle, the funnel develops also in December but it regresses at the theoretical time of ovulation. The anterior tubal part of the oviduct develops at the beginning of the cycle. The connective tissue has developed, vascularized and some crests are observed in a direction to the lumen. Around the lumen, the simple undifferentiated columnar epithelium becomes ciliated with gland cells between the crests. In December-January, some cells with sulfated mucus are found between the ciliated cells. At the beginning of pregnancy, this anterior part regresses and remains thin. At the beginning of the second year of the sexual cycle (October), the oviduct again prepares for pregnancy, but it degenerates at the theoretical period of ovulation. The uterine part has increased before ovulation. The wall first bordered with undifferentiated epithelium develops with cells equipped with microvilli, rounded ciliated cells and some gland cells with acid mucus. The well-developed connective tissue forms vascularized crests. At ovulation and at the beginning of pregnancy, a lot of cells become secretory, producing acidic mucus which covered ciliated cells. As the embryos grow, the uterine wall becomes distended, the secretions of gland cells reduce, and the cells become degraded. At the end of pregnancy, all the epithelial cells have degenerated and the connective tissue remains nude. The epithelium of the intra-uterine embryo's gills is narrowly applied against this connective tissue. After parturition, the uterine lumen becomes narrow and bordered with an undifferentiated tissue. A new differentiation of the uterus is observed at the beginning of the second year. The uterine wall regressed at the theoretical period of ovulation. In the following October, a new biennial cycle starts.

Apoptosis and proliferation in the oviduct

In *B. taitanus*, the percentage of proliferating cells visualized with Ki67 antibody was the highest in September-October (period of preparation to the breeding) and November-February (breeding), and the lowest in March-August (period of quiescence). The percentage of apoptotic cells visualized with TUNEL method or "Apostain" antibody, was significant throughout the year. The balance between proliferating and apoptotic cells varies throughout the year according to the part of the oviduct. During March-August, the apoptotic cells are more abundant than proliferating ones in the pars recta where they are lesser abundant during the periods of preparation and breeding (15% to 22.4%). In pars convoluted, the total number of proliferating cells fluctuates between the periods of rest and preparation (22% to 50%). The variations of the different cell types have been particularly investigated. In pars utera, the number of proliferating secretory cells,

the most represented epithelial cells in this part, increases after the period of quiescence (0% to 38%), and the number of apoptotic cells increases after the period of preparation to breeding (45% to 56%). During March-August (quiescence), the relative number of proliferative secretory cells is lower (about zero) than the relative number of apoptotic cells (50%). In pars convoluta and pars utera, no Ki-67-positive secretory cells are observed during the period of quiescence; they are visualized with the increasing number at next periods of the cycle. In this part of the oviduct, both proliferating and apoptotic cells are more frequent over the breeding period than over the preparation period, and more frequent over the preparation period than over the resting period [93].

At the sexual rest period and at the preparation for breeding, the goblet cells found in pars convoluta are not proliferating. During the period of reproduction, the percentage of Ki-67 positive goblet cells increases significantly throughout the year. The percentage of apoptotic goblet cells decreases progressively during these same periods. So, in these parts of the oviduct, apoptosis and proliferation increase after a regression during sexual rest. That can be reliable to the holocrine activity of these gland cells at breeding, the secretion of which constitutes the egg gangues. The gangue surrounding the egg is composed of two layers: an inner part situated against the egg which composition is the same glycoproteic granulations found in the secretory cells of the pars convoluta, and an external layer the composition of which being the same granulations found in the secretory cells of pars utera [94]. The ciliated and goblet cells facilitate the transit of eggs after fertilization occurring in the anterior part of the oviduct, like in other caecilian species [97,98].

In *T. compressicauda*, the proliferating cells were visualized with Ki67 antibody, and the proliferating indexes calculated for connective cells, and different epithelial cells (goblet, secretory, ciliated). In pregnant females, the proliferation indexes are comprised between 7% (connective cells) and 37% (epithelial cells). During the year of sexual rest and the period of quiescence, these indexes strongly decrease. In non-pregnant females, all the cells belonging to all tissues experience a proliferation index near zero.

In the uterus, during preparation for reproduction, indexes are 12.5% for connective cells and about 36% for epithelial cells. During the breeding period, the proliferation indexes are comprised between 20 % and 35% according to the cell type (connective cells, secretory cells, ciliated cells). At quiescence, these indexes decrease [94].

Apoptotic cells were detected with the use of the TUNEL method or "Apostain" antibody. In the tubal (anterior) part of the oviduct, during the phase of preparation (October), the percentage of apoptotic cells is comprised between 20% and 30%, whatever the cell type studied. During the breeding period, apoptosis affects about a third of these cells. During the year of quiescence, apoptosis affects a lesser number of cells. At the end of each year, the percentage of apoptotic cells increases. At the breeding season, proliferation rates are observed close to those of apoptosis throughout the genital tract, suggesting a turnover of cells in all tissues.

In the uterus, at the end of each year of the cycle, apoptosis affects about 45% of connective cells, and 25% to 33% epithelial cells (secretory and ciliated cells). In pregnant females, the percentage of apoptosis decreases. Programmed death affects more connective cells (36%) than epithelial ones (secretory and ciliated cells). During quiescence, apoptosis is also low in connective cells, 18.4% of secretory cells as well as secretory and ciliated cells.

These variations can be related to the reproductive physiology of the animal. At pregnancy, the connective tissue of the uterus appears rather in cell regression, while the epithelium multiplies. These variations are certainly linked to the trophic functions ensured by the female. A similar phenomenon has been already studied in some salamanders [99]. In *T. compressicauda*, the embryos feed secretions of the epithelium, they abrade it. After this phase of feeding, the embryos become applied against the uterine wall. Their gills become vesiculous, narrowly applied against the connective tissue of the uterine wall, forming a placenta-like structure [100]. Therefore, the embryos eroding the uterine wall might be the cause of the slowing apoptosis and increased proliferation.

In a general manner, the apoptosis/proliferation balance was favorable to apoptosis at sexual rest except for the lamina propria of the uterus. During the preparation for breeding, the apoptosis/proliferation balance was favorable to proliferation. These cell phenomena are consistent with the evolution of the oviduct throughout the reproductive cycle. The primary phenomenon indicating the starting-up of the development of the genital tract is certainly the increase in cell number. But apoptosis is also involved in the remodeling of the oviduct. In non-pregnant females, the genital tract also presented proliferation and apoptosis, consequent to the lack of corpora lutea.

These variations are under the hormonal control of the ovary and pituitary gland [101-103]. These observations suggest a different regulation in comparison to the seasonal sexual rest.

Sexual cycles and hormonal regulation in *T. compressicauda*

Among the species well-studied, *T. compressicauda* has been the subject of research devoted to studying the endocrinal regulation of sexual cycles. In females, the study of the pituitary gland showed that gonadotrophic and lactotrophic cells are narrowly linked to the sexual cycles. These cells increase in size during the phase of preparation to reproduction then they reach a maximal value at ovulation (February). During pregnancy, the cells decrease progressively to reach a minimal value at parturition. A new increase is observed at the new period of reproduction but after having reached a new maximal value at the theoretical period of ovulation, they strongly decrease. A new cycle begins at the end of this second year. An in situ hybridization study shows an increase of mRNAs encoding PRL at the onset of pregnancy, and RNAs encoding for PRL receptors were visualized in the corpora lutea, showing a direct relationship between corpora lutea and pituitary gland.

In ovaries, 17β estradiol, estrone, and progesterone were researched throughout the sexual cycle. At the phase of reproduction preparation, the granulosa cells surrounding the vitellogenic oocytes become voluminous, with the presence of estrogenic hormones. At pregnancy, after ovulation, the empty follicles bring on corpora lutea, the structure, and evolution of which having well studied previously. These corpora lutea become vascularized and the presence of progesterone is detected. Like previously said, the cells presented also mRNAs encoding for PRL receptors. At parturition, corpora lutea regressed. At the second year of the cycle, the oocytes enter in vitellogenesis and follicles develop. But at the theoretical period of ovulation, the vitellogenic oocytes degenerate and follicles became atretic.

Animals live in swamped savanna in the rainy season and in low-level water or sometimes buried in the muddy ground in a dry season. When animals are submitted in an artificial seasonal cycle without any

period of exondation, the female reproductive cycles are disrupted, becoming no-synchronized with males cycles [103]. The kidneys show morphometrical and histological variations according to the season [104], mesotocin and vasotocin are present in the brain [104], and their receptors are observed in the kidney [104]. In several species, a link between the hormones of hydromineral regulation and breeding has been documented [105-109]. The fact that *T. compressicauda* is submitted to a cycle of seasons permits to use it as a model to search a link between hydromineral regulation and reproduction [110].

The hormonal regulation of oviduct variation during the sexual cycle has been also investigated in *B. taitanus*. In this species, the presence of 17β estradiol, estrone and progesterone has been visualized using the immunohistochemical method. The distribution of these hormones is comparable to that of *T. compressicauda* [111]. The presence of the receptor of 17β estradiol has been revealed in both ovaries and oviducts. In ovaries, α and β receptors have been detected in somatic cells with different ratios between the two receptors, according to the cell type. This ratio also varied throughout the sexual cycle, suggesting the existence of a link between the β receptor involved in vitellogenesis maintenance throughout the year and the seasonal modulations of granulosa development.

In the oviducts, the regulation of cyclic variations is under the control of ovarian hormones. α and β receptors of 17ß estradiol have been visualized in both nucleus and cytoplasm of cells belonging to various tissues. So, a pleiotropic action of 17ß estradiol can be highlighted, as well as the existence of action not only genomic but also metabolic and probably epigenetic.

Conclusion

To conclude, it is obvious that amphibians are interesting models to understand several physiological phenomena. Since a long time, amphibians have been models to studying anatomical, histological aspects of development or differentiation of sexual organs, then hormonal regulation of metamorphosis or differentiation/ dedifferentiation of sexual organs. These same animals are also models to study the effects of environmental factors. Today, it seems important to consider the epigenetic factors which certainly can explain relationships between the variations of organs (development, cyclic variations) and the variations of environmental factors. Amphibians performing a development directly in the external environment, or being submitted to the variations of the season to breed, are excellent models to study these more recent aspects of gene expression throughout these physiological phenomena.

Amphibian species may be proposed such as models to understand the importance of cell proliferation, differentiation, and death. Here we show specific aspects of amphibian development such as the regulation of proliferation, differentiation, and apoptosis during metamorphosis and also very particular aspects of the same processes during the preparation for reproduction, notably in Caecilians. Caecilians, also called Gymnophionans, as lengthening, burrowing or aquatic species, display a specific adaptation to their local environment which is illustrated by modifications in genital organs in response to environmental changes. The genital changes include a change in cell count, in cell type, in tissue differentiation, and in cell and/or organ morphology, as it was described here for the oviducts for example. The genital changes recapitulate a seasonal cycle in females which makes the oviduct a good candidate to address the mechanisms underlying the interplay of environmental features (humidity, temperature, food), metabolic regulation (hydromineral balance) and cell or tissue adaptation (preparation for breeding).

Epigenetics is known to be the molecular exploration of the variations occurring in response to environmental change without changing the DNA structure, these variations being heritable via mitosis or meiosis and reversible [112]. Herpetofauna (amphibians and reptiles) represent important sentinel and indicator species for environmental and ecosystem health. Environmental stimuli influence gene activity, and there is growing evidence demonstrating that an important mechanism is through modulation of the epigenome [113]. Moreover, methylation has been shown to help to reveal epigenetic plasticity in response to developmental or environmental constraints [114,115].

Cytosine methylation (5mC) is involved in three vital biological processes in Mammals: embryogenesis, genomic imprinting and the regulation of transcription [116-119]. 5-hydroxymethylcytosine (5hmC). Recently detected in mice, rats, rabbits, and cattle [120-124] and also in amphibians [125], is reported to regulate developmental processes, neurogenesis and cellular differentiation [126,127]. Yet, if the contribution of epigenetics in a developmental context is well established, which genomic sites are changing and what are the contributions of methylation and hydroxymethylation to the epigenetic plasticity in response to seasonal change is still poorly documented. Here we found in Caecilians, a model to study epigenetic plasticity involved in differentiation and morphologic modifications in response to seasonal change. Studying how epigenetics contribute to the adaptation of organisms has now become a priority in order to bring new insights into the understanding of epigenetic adaptability in Vertebrates in a period of climatic change.

Acknowledgment

The works cited have been supported by the Lyon Catholic University. The authors thank Ms. Marie-Thérèse Laurent and Ms. Caroline Bouchot for their technical assistance. They also thank the students who participated in the study of punctual aspects of reproduction and development of the amphibian models.

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