
Pond Snail Reproduction as Model in the Environmental Risk Assessment: Reality and Doubts

Zsolt Pirger, Zita Zrinyi, Gábor Maász,
Éva Molnár and Tibor Kiss

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Abstract

In European limnetic systems, the most relevant endocrine-disrupting chemicals (EDCs) of steroid type are the natural and synthetic hormones, phytosterols, pesticides, biocides and other chemicals produced by the plastic industry. Their presence in aquatic ecosystems represents a potentially adverse environmental and public health impact. Furthermore, this is a warning signal that the current handling of pharmaceuticals needs to be further improved. Nowadays, it has become clear that EDCs have specific disturbing effects on the neuroendocrine system of invertebrate and vertebrate aquatic animals, particularly gastropods. Among a latter, pond snail (*Lymnaea stagnalis*) has been used as the first aquatic non-arthropod test organism in studying the effect of EDCs because they are sensitive to various anthropogenic steroids, like progestogens. Investigating a variety of reproductive endpoints of *Lymnaea*, such as fecundity, oocyte production, egg mass production, the quality of egg masses, the shell size in development and after egg-laying, the time window of cell division in the offspring, the metabolite content of single-cell zygotes and egg albumen has concluded that progestogen contaminations in water are detrimental for reproduction and early stage development of *Lymnaea*. This chapter is an attempt to show whether *Lymnaea* reproduction, despite many altering reproductive endpoints, is a suitable model for environmental risk assessment or not.

Keywords: endocrine-disrupting chemicals, progestogens, molluscs, *Lymnaea stagnalis*, reproduction model

1. Introduction

In the last few years, it has become clear that a wide variety of environmental contaminants have specific effects on neuroendocrine system of aquatic species. The frequent detection of

endocrine-disrupting chemicals (EDCs) in the aquatic environment and a high consumption of contraceptives all over the world reflect a rapidly growing concern on their environmental impact. EDCs interfere with the body's endocrine system mimicking or partly mimicking naturally occurring hormones in the body and induce adverse developmental, reproductive, neurological (cognitive and behavior) and immune effects in both humans and wildlife [1]. In addition, the high frequencies of detection of these contaminants in aquatic environments and the incomplete removal of them during passage through sewage treatment plants may pose the greatest risk during prenatal and early postnatal development when organ and neural systems are developing. The increasing and continuous occurrence of steroidal estrogen and progestogen compounds in the environment can lead to toxicological effects on non-target organisms, therefore, it is important on the whole to assess the environmental risk posed by these contaminants.

Molluscs like gastropods and bivalves have been used as non-target model organisms in studying environmental contamination for a long. They proved to be effective model animals because they are ubiquitous, have highly conserved control and regulatory biochemical pathways that are often homologous to vertebrate systems and they are extremely sensitive to anthropogenic inputs [2–4]. For example, the bivalves, by virtue their ability to accumulate toxic substances (due to their sessile and filtering life style) in their body are considered as excellent indicators of ecosystem health [5]. Furthermore, molluscs are ecologically crucial organisms, which are essential to the biosphere and to the human economy. They are the second most diverse animal group (10 taxonomic classes) encompassing more than 400,000 species, they are ecologically and commercially important as food and non-food resources. Among them terrestrial gastropods are destructive agricultural pests causing economic damage to a wide variety of plants including horticulture, field crops and forestry. In addition they are of importance in medical and veterinary practice, since they serve as intermediate hosts for several human and animal diseases, such as schistosomiasis and helminth diseases [6]. Both terrestrial (e.g. *Helix pomatia*), marine (e.g. *Aplysia californica*) and freshwater (e.g. *Lymnaea stagnalis*) snails have proved to be excellent models, due to their “simple” nervous system, in neurophysiology and behavioral ecology [7–12]. Gastropod model organisms play an important role for immunology [13], reproductive and developmental biology (which is facilitated by several genome and transcriptome projects that are currently underway) [14–16], neurobiology, especially on learning and memory formation [17–22]. Some species, in particular simple pond snail (*Lymnaea stagnalis*) have been widely applied in pollution biomonitoring programs, and widely used in a variety of ecotoxicological studies [23–28]. Based on earlier investigations the reproduction test of *L. stagnalis* was officially approved by the national coordinators of the Organization for Economic Cooperation and Development (OECD) member countries as test guidelines. *L. stagnalis* and the New Zealand mudsnail (*Potamopyrgus antipodarum*) have been the first aquatic non-arthropod-tests, which were successfully validated within the Conceptual Framework for Endocrine Disrupters [3, 29, 30]. Therefore, in this chapter one of the most relevant mollusc of European limnetic systems, the hermaphroditic *L. stagnalis* is particularly presented to model the various physiological effects on its reproductive and developmental parameters eliciting by acute or chronic exposures of endocrine-disrupting substances. A variety of endpoints are assessed and collected, including fecundity, oocyte production, egg mass

production, the quality of egg masses, the shell size in successive development and following egg-laying, the time window of cell division in the offspring, the metabolite content of single-cell zygotes and egg albumen before and after the treatment of parents.

It has been shown that recent research aims to combine molecular level investigation with cellular, organismal, behavior and environmental research. In this chapter, an attempt is made to summarize data particularly obtained on *L. stagnalis* so far, and to discuss the molecular mechanisms, the functional and ecological consequence of EDCs and the advantages of snail preparations as tools for ecotoxicological research. Comparison of the data obtained on molluscs with those obtained on the lower vertebrates, will definitively contribute to the better understanding of the impact caused by EDCs, like steroid hormones, present in our environment.

2. Steroid type EDCs in the aquatic environment

The release of human pharmaceuticals (as xenobiotics) into aquatic ecosystems is a serious environmental risk which results in an acute and chronic contamination of non-target invertebrate (e.g. molluscs) and vertebrate (e.g. fish) freshwater organisms [31]. Among the most critical environment contaminants are EDCs, which are defined as an exogenous substance that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism. It is concluded that endocrine disruption is not considered a toxicological end point per se but a functional change that may lead to adverse effects in both non-target and target organisms, as well. EDCs act as agonist or antagonists at multiple sites via complex mechanisms of action including: receptor-mediated mechanisms, synthesis and/or metabolism of hormones, neuropeptides and neurotransmitters, as well as transport pathways [32].

In European limnetic system, the most relevant steroid type EDCs are follows: natural (e.g. progesterone, estradiol, testosterone [33–35] and synthetic (e.g. drospirenone, levonorgestrel, ethinylestradiol, cyproterone acetate (CPA), t-methyltestosterone [23, 33–35]) hormones, phytoosterols (e.g. β -sitosterol [23]), pesticides (e.g. octylphenol, chlordecone [35, 36]), fungicides (e.g. vinclozolin (VZ), pyraclostrobin [25, 28]), biocides (e.g. tributyltin [23, 36]) and other chemicals produced in the plastic industry (e.g. bisphenol A [36]). One of the most cited examples to steroidal EDCs is the tributyltin (TBT) in molluscs. It caused imposex and intersex development as two masculinization phenomena in more than 260 species of gastropod worldwide, and severe losses of invertebrate biodiversity in waters [5, 37]. Several studies on perturbations of mollusc reproduction following exposure to low concentrations (ng/L range) of steroid type EDCs have already been reported. These more recent studies collectively provide evidence for possible detrimental effects of steroidal EDCs on *L. stagnalis* reproduction and embryonic development. However, the underlying mechanisms between exposure to EDCs and a variety of biologic outcomes, their potential long-term side effects of these molecules on molluscs remain largely unknown. This book chapter is mainly focused on synthetic steroids because they have become one of the most harmful pharmaceutical pollutants in molluscs.

Synthetic steroids, like estrogens and progestogens, are potent endocrine disrupters, which can modify diverse physiological, hormonal and behavioral processes in freshwater species,

and subsequently affect their capacity to reproduce, develop and grow [38, 39]. Estrogens and progestogens in combination are widely used as synthetic oral contraceptives (SOCs) [40]. SOC residues or their metabolites are eliminated from the human body unchanged or in the form of active metabolites in a remarkable amount [41, 42]. These biologically active agents enter into the waste water treatment plants (WWTP) where the generally applied treatment process is not suitable to eliminate them perfectly [42–45]. Consequently, synthetic steroid hormone residues enter the aquatic environment (e.g. surface waters) mainly through cleaned effluents. The first review, which describes the presence of estrogen and progestogen hormones in natural surface waters was published by Richardson and Bowron [46]. In fact, very few pharmaceutical chemicals were identified due to the limitations of the early gas chromatography and HPLC techniques. The development of analytical techniques (e.g. liquid chromatographic-mass spectrometric method with solid-phase extraction, see later) decreased the limit of detection, resulting in an increasing number of detectable SOC residues in surface and ground water, as well [47, 48]. Nowadays, their reported presence are in a concentration range from a few ng/L to often tens or hundreds of ng/L (estrogens: 0.20–480.00 ng/L, progestogens: 0.07–22.20 ng/L) in surface waters [47, 49–51]. The catchment area of the largest shallow lake of Central Europe is a habitat of several molluscs (e.g. *L. stagnalis*, *Anodonta cygnaea*, *Dreissena polymorpha*) and fish species (e.g. *Rutilus rutilus*, *Alburnus alburnus*, *Abramis brama*, *Carassius carassius*, *Cyprinus carpio*, *Perca fluviatilis*), where the estrogen and progestogen concentrations were found between 0.07–0.68 ng/L and 0.23–13.67 ng/L, respectively [33, 34]. The presence of steroid hormones has also been found in the drinking water, which is a warning sign that the current handling of pharmaceuticals may lead to future global human health problems [52–56]. It has already been described that exogenous steroid contaminations have wide range genotoxicity, neurotoxicity and germ cell-damaging effects in humans. For example, ethinylestradiol may modify brain structure, function, and consequently, behaviors pattern during the female brain development [57]. Furthermore, accumulating evidence suggest that human exposure to steroids is related to the impairment of male reproductive function (e.g. decreased sperm number) and can interrupt other hormonally regulated metabolic processes, particularly if exposure occurs during early development [58].

2.1. Methods in detection of steroidal EDCs

Measurements of multi-residue analysis require a rapid, sensitive, robust and reliable method with fast response time (high-throughput). These analytical measurements are essentially determined by two crucial things, one is the limit of detection, and the other is the sample (matrix) complexity. The subject of detection limits in analytical chemistry has improved since the 1970s and these resulted that the amount of detectable analytes, such as EDCs, are decreased [59–62]. Nowadays, the mass spectrometry based methods are extended and their detection limits are almost low ppm or ppb, which are below the environmentally relevant concentrations at the time. Other problem with the detection and quantification of an analyte can result from different matrix effects, sample concentration or other conditions, such as instrument sensitivity and reagent purity. In general, these matrices have different type of waters (e.g. wastewater influents or effluents, ground-, surface- and tap waters) and various solid samples (sediment, sludge, biological matrices). Sample preparation techniques can enhance the performance results for better recovery, increased sensitivity and lower detection limits [63].

Multi-residue analysis, as a field of study encompassing steroid EDCs residue analysis, has made considerable advances regarding selectivity and detection limits. Before analytical procedures, in order to keep track levels of EDCs, it is recommended that (e.g. deuterated) internal standards are added to the water or solid samples [64]. In general, there are several extraction methods, such as liquid-liquid extraction (LLE), solid-phase extraction (SPE), solid-phase micro-extraction (SPME), stir-bar sorptive extraction (SBSE), selective pressurized liquid extraction (SPLE), Soxhlet extraction (SE), ultrasonic extraction (USE), microwave-assisted extraction (MAE) and accelerated solvent extraction (ASE) [65–68]. The majority of current analytical methods for separation and detection of various steroidal EDCs, for example, use liquid chromatography-tandem mass spectrometry (LC-MS/MS) because its versatility, specificity and selectivity are very well [69]. Other possibility to detection and quantitative measurement of steroidal EDCs is also offered by gas chromatography (GC) with electron capture detection and confirmation by MS [70].

In case of water samples, the main steps of analytical methods are the filtration (e.g. glass microfiber filters), extraction and purification (e.g. SPE), finally quantitative measured by using LC-MS/MS. Generally, around 0.1 ng/L limit of quantification (LOQ) value are achieved [33, 34, 71, 72]. The detection of steroid EDCs from various solid samples are complicated because more sample preparation steps are required (drying, homogenization, destruction, extraction and purification). The most commonly applied extraction methods are USE, MAE and SPLE for solid environmental matrices, such as sediment or biological tissues [64–68]. After extraction procedure, off-line SPE and LC-MS/MS are utilized for EDCs analysis [64, 73, 74].

2.2. Progestogens as neuroendocrine disruptors: an outlook on the world of fish

Together with synthetic estrogenic steroids, progestogens are among the most important group of environmental pharmaceuticals of concern. A large number of studies investigating the occurrence and effects of natural and synthetic estrogen hormones (e.g. ethinylestradiol, estradiol, estrone and estriol), and the risk is now well documented [47, 49, 75, 76]. Several studies have also been conducted on the risk related to anti-androgens [77], but contrast to these, surprisingly, relatively few data are published about the occurrence of progestogens in different waters [34, 41, 49] and mainly their neuroendocrine effects on non-target freshwater organisms, including particularly invertebrates [49].

Progesterone (PRG) is an endogenous steroid hormone involved in the female menstrual cycle, pregnancy and the embryogenesis of humans and other vertebrate species. In turn progestins are a group of natural and synthetic molecules that have effects similar to those exerted by PRG. The endogenous PRG and its analogue progestins together are generally referred to as progestogens (or gestagens). The most important and frequent synthetic progestogens are the follows drospirenone (DRO), levonorgestrel (LNG), gestodene (GES), norethindrone (NET) and ciproterone acetate (CPA). The progestogens that are used in hormonal contraceptives are LNG (e.g. Alesse, Trivora-28, Plan B, Mirena), DRO (e.g. Yasmin, Yasminelle), GES (e.g. Femodene) and CPA (e.g. Diane-35, Dianette). There are approximately 20 different progestogens used in human and veterinary medicine. Despite significant use, their ecotoxicological implications are poorly understood in environment. According to Fent, only about 50% of the progestogens in use have been analyzed for their environmental occurrence and effects in aquatic organisms [49].

For example, in fish, the main natural progesterin is $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP). In females, DHP is responsible for maturation of oocytes [78] and ovulation [79], while in males it is involved in spermiation and sperm motility [80]. Synthetic progestogen contaminations altered hormone levels [81], induced transcriptional effects in adults [82] and embryos [83], altered sex development and induced development of male secondary sexual characteristics in female fish [81, 84]. Therefore, there are evidences that progestogen contamination interferes with endogen steroids and adversely affect fish reproduction. According to literature data, LNG and GES significantly reduce egg production in fathead minnow (*Pimephales promelas*) [81, 84]. At environmental ng/L concentrations, progestogens could interfere with natural pheromones, therefore also impair the physiological responses and spawning behavior in fish [85, 86]. In addition, based on earlier work it has been shown that chronic exposure to a mixture of PRG, LNG, DRO induce complex molecular changes both in brain, liver and serum of roach (*Rutilus rutilus*) [87]. Collectively in vertebrates, progestogens activate nuclear PRG receptors [88], but also may activate other steroid receptors, such as the androgen, estrogen, glucocorticoid and mineralocorticoid receptors, exerting combinations of progestogenic, (anti)androgenic, (anti)estrogenic, glucocorticoidogenic and anti-mineralocorticoidogenic effects [89].

3. Molluscs as “possible and valuable” model animals in environmental tests

3.1. Sex steroid-like receptors in molluscs

PRG receptor immunoreactive elements were identified in the reproductive system of the female *Octopus vulgaris* [90]. According to Tosti, the PRG receptors are physiologically active because the external application of PRG stimulates the activation of spermatozoa in *Octopus* [91]. In contrast to cephalopods, no progestogen-like receptors have been identified in snails so far. The androgen-like receptor immunopositive cells has already been described in ovotestis of *Biomphalaria alexandrina*, and there is some (inferred) evidence of a role for androgen-like molecules in the reproductive cycle of molluscs [92–94]. But the fact is that homolog or orthologue sequences were not identified in molluscs despite investigations searching for the androgen receptor gene [95]. In contrast, estrogen receptor orthologues have previously been reported in number of freshwater and marine molluscs, such as *Aplysia californica*, *Biomphalaria glabrata*, *Bithynia tentaculata*, *Marisa cornuarietis*, *Potamopyrgus antipodarum*, *Nucella lapillus*, *Chlamys farreri*, *Crassostrea gigas*, *Lottia gigantean*, *Mytilus edulis*, *Octopus vulgaris* and *Sepiella japonica* [93, 96, 97]. The existence of an estrogen or estrogen-related receptor has been confirmed in *Lymnaeidae* sp. (e.g. *L. ollula*) [98], however in *L. stagnalis* is not investigated so far. The amino acid sequence of endocrine receptor of oyster (*Saccostrea glomerata*) contains a DNA-binding domain and a ligand-binding domain which are conserved among vertebrate endocrine receptors [99]. However, it is worth to mention that real function of identified estrogen receptors are questionable at present, because ligand studies show that the receptor homolog is non-sensitive to estrogen in the oyster, for example [100]. Even so, many researchers speculate that the most steroid pollutants act through the estrogen or androgen-like receptors in molluscs [93]. It is also known that the endocrine effect of TBT (steroid biocide) appear

through binding to a nuclear receptor (the retinoid × receptor) in *Nucella lapillus*. The natural ligand (9-cis-retinoic acid) of the retinoid × receptor induces similar imposex in females of *N. lapillus* than TBT at similar concentration [101, 102]. Despite the contradictory observations and opinions about the presence of steroid-like receptors in molluscs, as well as the limited genetic evidence for steroid receptors, binding proteins for classical vertebrate-type steroids have been described. However, it has not yet been demonstrated that this binding is coupled to an endocrine biological response. Some researchers speculate that vertebrate-like steroids, such as estrogen, can also/just act through non-genomic mechanisms in mollusc. Non-genomic action of steroids are expressed through cell surface membrane receptors (not nuclear receptors) and in this case they also can results direct local “ionotropic” effects (e.g. modification ion fluxes) and/or they can activate second messenger kinase cascade system during “metabotropic” pathway (e.g. cAMP-MAPK-PKC) [103, 104].

3.2. Endocrine steroid system of molluscs: evidences and questions

Despite many published studies reporting presence of vertebrate-like sex steroids, steroidogenic enzymes and steroid receptors in molluscs, the endocrine system is the most unclear and contradictory topic of molluscan research. It is generally accepted that vertebrate-type steroids, as PRG, estradiol or testosterone, are presented in various molluscan tissues (e.g. gonads, haemolymph) and they are physiologically potent molecules performing hormonal functions. Regarding their endogenous biosynthesis, evidences are contradictory. At present is unknown whether vertebrate-type sex steroids are formed endogenously during steroidogenesis or they are taken up from their environment through the feeding because it is known that many plant species contain vertebrate-like sex steroids [105]. Since PRG, estradiol and testosterone as functional hormones in mollusc are the same as those of vertebrates, and vertebrates continuously excrete them not just via urine and feces, but via their body surface or gills (in fish), the other possibility is that observed “molluscan” steroids just come from contamination [95, 106]. At the same time, several papers have been published presenting evidence of steroidogenic activity and steroid metabolism in molluscs [107, 108]. For example, beside other metabolic enzymes (e.g. 5 α -reductase, sulfotransferase, and acyl-CoA acyltransferases) the occurrence and activity of two key steroidogenic enzymes 3 α / β -hydroxysteroid dehydrogenase (HSD) and 17 β -HSD are presented in several molluscan species. The 3 α / β -HSD is the key enzyme in conversion of progesterone (P5) to PRG. This enzyme has been described in *Ariolimax californicus*, *Aplysia depilans*, *Helix pomatia*, *Mytilus edulis* and *Octopus vulgaris*. The 17 β -HSD is crucial molecule in the last step of steroid syntheses and the primary metabolism. The 17 β -HSD catalyzes the interconversion of androstenedione to testosterone, estrone to 17 β -estradiol and androstenedione to dihydrotestosterone. The 17 β -HSD enzyme has been detected in many snails (e.g. *Marisa cornuarietis*, *Ilyanassa obsoleta*, *Hexaplex trunculus*, *Bolinus brandaris* and *Helix aspersa*), bivalves (e.g. *Crassostrea gigas*, *Crassostrea virginica*, *M. edulis*, *M. galloprovincialis*, *Ruditapes decussate* and *Patinopecten yessoensis*) and cephalopods (e.g. *Sepia officinalis* and *O. vulgaris*) so far. These observations comprise a series of indications about the existence of steroidogenesis in different molluscs [107, 108]. At present, no data are available about the expression of key enzymes in *L. stagnalis*, however the cholesterol which is the direct precursor of P5 has been described in its neurons [109]. According to literature

data, *L. stagnalis* was able to transform PRG from injected labeled P5 [110]. The P5 is a key molecule in the biosynthetic pathway of main vertebrate steroids, such as PRG, 17 β -estradiol and testosterone which have also been proposed as functional hormones in molluscs [95].

Steroidogenesis and steroid metabolism play an important role in the regulation of endogenous steroid level in molluscs. As a result of their endogenous biosynthesis, active P5 (e.g. in *M. edulis*, *Astacus leptodactylus* and *Nephtrops norvegicus*), PRG (e.g. in *M. edulis*, *Mya arenaria* and *O. vulgaris*), androstenedione (e.g. in *M. edulis*, *H. aspersa*, *A. leptodactylus* and *Neomysis integer*), estron (e.g. in *M. edulis*, *Arion ater rufus*, *H. aspersa* and *Asterias rubens*) and testosterone (e.g. in *M. edulis*, *Arion ater rufus*, *A. leptodactylus* and *N. integer*) have been described in several molluscan species [108]. In addition, it has been published that the physiological concentration of these sex steroids are related to changes in the reproductive cycle and their level are higher in one sex than other, or their level are changed during EDC contamination. Furthermore, another vertebrate-type hormone, the gonadotropin-releasing hormone (GnRH) stimulates the synthesis and release of "molluscan" sex steroids from the gonads, and elicited contractions of the oviduct, for example, in cephalopods. This result suggests that octopus-GnRH induces the gonadal maturation and oviposition by regulating sex steroidogenesis [111]. GnRH-like hormone has also been identified in two freshwater snails, *Helisoma trivolvis* and *L. stagnalis*, presumably with a similar control function than in cephalopods [112]. From a phylogenetic point of view, observations of steroidogenesis and vertebrate-type steroids are very interesting because they indicate a common origin of a sex hormonal system between molluscs and vertebrates. However, much more information is needed to fully understand the physiological function of sex steroid hormones in molluscs. But also noticeable that according to valuable Scott's reviews, despite many studies starting over 55 years ago, these data are questionable from several reasons. For example, the mollusc genome (so far known) does not contain genes for key enzymes that are necessary to transform cholesterol (precursor molecule in steroid biosynthesis) [95, 106].

3.3. Reproductive system and behavior of *L. stagnalis*

The reproductive biology of *L. stagnalis* has been well-studied [14, 15, 113, 114]. It is a hermaphrodite species, but during mating behavior one individual acts as male and the other as female. The snail playing the male role climbs on the shell of the prospective female, moves over the shell in a counter-clockwise direction until he reaches the area of the female gonophore. The preputium (muscular structure that surrounds the penis) is then partially everted through the male pore. This is followed by probing for the female pore by the preputium, insertion of this organ into this pore followed by penis eversion and intromission. Each of the four stages prior to intromission is variable in duration but the intromission is more constant. The whole mating behavior may last for several hours. During oviposition (egg-laying), masses containing 50–100 eggs embedded in a gelatinous mass are deposited on the substrate, from which juvenile snails of adult form emerge following about 10 days of intracapsular embryogenic development, without any free-living larval stages [113, 114]. Egg-laying consists of a sequence of behavioral events beginning with a rest period when the animal ceases to locomote, then a turning phase characterized by counter-clockwise shell movements and high frequency rasping to clean the substrate, followed by oviposition and a final phase

called inspection when the snail moves along the length of the egg mass brushing it with lips and tentacles. Resting and turning last for about an hour each, oviposition 10 minutes and inspection about 2 minutes. Egg-laying in *L. stagnalis* is an example of a complex behavior that is triggered by the release of multiple neuropeptide transmitters from neuroendocrine centers within the central nervous system that act on other neural circuits controlling egg-laying behavior [12]. Whether the action of described sex steroids is receptor-mediated or not is unclear in *L. stagnalis* at present. But it is a fact that vertebrate-like sex steroids might have a key role in reproduction in snails. Temporal variation in some steroid titers that coincide with reproductive stages have been observed.

3.4. Progestogen effects in *L. stagnalis*

Cyproterone acetate (CPA) is a commonly used synthetic progestogen compound in oral contraceptives, which also has anti-androgen effects in vertebrates. The vinclozolin (VZ) is mainly known as anti-androgen, which can also bind to estrogen and progestogen receptors in vertebrates. Using these progestogens, Ducrot and Giusti published no significant difference in shell length of adult *L. stagnalis* between control and treated groups after 21 days of exposure to any of the tested chemicals [25, 27]. However, Ducrot observed slower growing among juveniles, sub-adults and young adults exposed to the highest concentration of VZ (2500 µg/L) during the first week, but partly recovered during the second and third weeks, so that growth pattern did not lead to a significant decrease in the mean shell size compared to control group. In Giusti's experiments, neither CPA (2–50 µg/L), nor VZ (10–240 ng/L) induced more than 10% mortality. According to Ducrot, significant mortality occurred in treated adult animals exposed to the highest concentration of VZ, whereas the feeding activity was stopped in this group. CPA and VZ had no significant effect on cumulated oviposition and fertility in adult snails in single chemical treatment approach, however 2 and 10 µg/L CPA as well as 10–240 ng/L VZ treatments induced a significant decrease in egg number per egg mass compared to control. However, in this experiment no clear concentration-response relationship was described [27, 106]. CPA, VZ and tributyltin (TBT) were also tested on other three gastropod species, *Marisa cornuarietis*, *Nucella lapillus* and *Nassarius reticulatus* in a chronic experiment. In this investigation, the snails were treated by nominal CPA concentration of 1.25 mg/L for 12 month or by nominal VZ concentration of 0.03–1.0 µg/L for up to 5 month. It was reported that no mortality at used concentrations, but a significant decrease in the length of the penis and accessory male sex organs was observed in both species [106, 115]. Since progestogen contaminations are expected to be found in the environment as mixtures, Zrinyi and her co-workers applied them together and environmentally relevant concentration range in *L. stagnalis* reproduction tests [116]. In a 10 ng/L eqi-concentration mixture of PRG, LNG, GES and DRO treatment approach resulted that the oocyte production of individual animals significantly changed in the treated group at the end of the 21-day long experiment. The number of laid oocyte per egg mass of individual animals shows continuous growth in the treated group week by week. Their number at the end of the third week was in average of twofold higher compared to the control. The control animals produced the same amount of oocytes weekly during experiment.

Beside egg number assessment, egg abnormalities can also be observe (e.g. polyembryonic egg, atrophied albumen, unfertilized oocyte or dead zygote in eggs), which refer to reproductive

status and can determine the egg quality. Egg quality showed no significant difference in CPA and VZ treatments, however polyembryony was the most frequent phenomena. A 3-week long CPA treatment resulted in significant increase of the frequency of polyembryony in concentration dependent manner from 2 $\mu\text{g/L}$ concentration [27]. In addition, the whole egg mass quality was also assessed in progesterone mixture treatment of adult snails with a three-graded scheme, which integrates the number of polyembryonic eggs with eggs containing dead zygotes. This endpoint resulted in a significant difference by the first week (**Figure 1**), but did not show difference at the end of a 3-week long experiment [116]. Based on published data, following the time window of early embryonic development in *L. stagnalis* could be able to another well endpoint for investigation of external progesterone contaminations. Progesterone mixture applied in environmental relevant concentration (10 ng/L) had significant effect on cell proliferation during early embryonic development. At the end of a 3-week long treatment period of the adult snails, the freshly laid zygotes were observed from the single-cell to the eight-cell stage. In zygotes obtained from hormone-treated adults, a significantly different accelerated cell proliferation could be noticed compared to controls, however the hatching time was unchanged [116]. In single-cell zygotes as well as egg albumen, a partial metabolomic analysis was also carried out using capillary microsampling combined ion mobility separation mass spectrometric technique. It was observed that the molecular composition of zygotes or egg albumen does not differ after steroid treatment of adults, but some semi-quantitative metabolic ratio (e.g. adenylate energy charge (AEC), redox ratio or hexose utilization) can express difference between the groups. These ratios could be used as marked endpoint in assessment of progesterone exposition in snails. The hexose utilization defined by UDP-hexNAc/UDP-hex ratio significantly decreased in single-cell zygote cytoplasm after a 3-week long progesterone mixture treatment of adults. This result partly could explain the observed accelerated cell division in zygotes obtained from treated parents. At the same time, AEC indicating the energy state of the cell was unchanged. This endpoint was significantly increased only in the albumen (obtained from treated adults) during the metamorphosis, which is the half-time of the average hatching time [116].



Figure 1. Evaluation of the egg mass quality in *Lymnaea*. Figure demonstrates the categories of the egg mass quality in the control and with the 10 ng/L progesterone-treated groups. The egg mass was presented in good (white), fair (gray) and poor (black) quality in the 1st, 2nd and 3rd weeks in both groups. On the 1st week, the egg mass quality was significantly different between the groups (Kruskal-Wallis $\text{Chi}^2 = 6.31$; $P < 0.05$; $n = 41$), while this was statistically not different in next 2 weeks (Kruskal-Wallis $\text{Chi}^2 = 3.65$; $P = 0.56$; $n = 50$ and $\text{Chi}^2 = 0.15$; $P = 0.70$; $n = 43$). $P < 0.05$ is signified by asterisk (*).

Based on several published data, we conclude that progestogen contaminations in water ecosystem are harmful for reproduction and early stage development of *L. stagnalis*. But, taken separately, progestogens might not present a risk for the snails, however since they are expected be found in the environment as mixtures (similar then in earlier experiment), there is a risk of additive or even synergistic effects [41]. Together with synthetic estrogenic steroids, progestogens are among the most important group of environmental pharmaceuticals of concern. However, in contrast to estrogens, progestogens have received only little attention so far and their environmental risks are not sufficiently known. Further investigations are needed to fully understand the synergistic effects of mixed progestogens or combined effects of progestogens and estrogens in freshwater organisms, such as molluscs.

3.5. *L. stagnalis* became a “real” test animal in EDC experiment

The OECD Conceptual Framework for Testing and Assessment of Endocrine Disruptors is available from 2004 (revised and completed with mollusc’s tests in 2012) [117]. This recommendation provides a guide with a five-level assessment but not indented to be a testing strategy of various EDCs. Another OECD reproductive toxicity test guideline with the pond snail *L. stagnalis* is also available from 2010 [25] and this optimized in 2016 using the steroidal TBT [30]. Several studies published data with different endpoints (number of egg mass, eggs, survival and shell size) recommended in OECD guidelines in progestogen exposure of *L. stagnalis* [23–28, 116]. Based on endpoint results coming from steroid, progestogen effects in snail reproduction, the pond snail, *L. stagnalis*, beside a mudsnail, *Potamopyrgus antipodarum*, has been the first aquatic non-arthropod-tests, which were successfully validated within the Conceptual Framework for Endocrine Disruptors [3].

4. General considerations: ecotoxicologist versus physiologist

Nowadays, we realized that a wide variety of environmental contaminants have specific effects on neuroendocrine system of aquatic species, including snails. For among them, *L. stagnalis* has been used as non-target model organisms in studying environmental contamination long time because they are sensitive to anthropogenic steroids, such as progestogens. Investigating a variety of reproductive endpoints, such as fecundity, oocyte production, egg mass production, the quality of egg masses, the shell size in development and after egg-laying, the time window of cell division in the offspring, the metabolite content of single-cell zygotes and egg albumen, it is concluded that progestogen contaminations in water are detrimental for reproduction and early stage development of *L. stagnalis*. Based on its endpoint results, the *L. stagnalis* has become the first aquatic non-arthropod-tests, validated successfully within the Conceptual Framework for Endocrine Disruptors. In this context, the proposed model is ecotoxicologically correct because it has well detectable effects. But if we are interested in physiological mechanisms of steroids (progestogen), many uncleared questions and contradictory observations are detected. For example, how progestogen contamination influences the *Lymnaea* reproduction is difficult to explain because progestogen and androgen

receptors until recently were not observed. At the same time, the identified estrogen receptor was found to be insensitive to estrogen. Whether estrogen binds to its receptor and the hormone-receptor complex remains inactive or it does not bind at all is unknown. Furthermore, according to some assumptions, the key enzymes for steroidogenesis are also missing in gastropods, therefore, the biosynthesis of vertebrate-type steroid hormones are questionable. If it is true, how can endogen “gastropod” steroids control the reproduction pathways?

We guess that some researchers did not consider that active state of many (terrestrial and freshwater) gastropod species depend on the season. For example, hibernation, aestivation or inactive state is an evolutionary mode of adaptation of animal species to unfavorable environmental conditions, such as low temperature or lack of food in autumn or winter. During the inactive state, normally no reproduction is observed in nature. This observation can be explained either by the low metabolism in unfavorable conditions (no steroid hormone synthesis) of the snails or vertebrate-type steroids cannot be taken up from environment through the feeding. Most of recent experiments on *Lymnaea* are performed on bred animals. It is possible that the evolutionary conserved seasonal (normal) rhythm of steroidogenesis and endogen level of steroids controlling reproduction will be damaged in artificial laboratory conditions. In this case, the steroids will be present at steady level instead of the normal wavering and the egg laying is continuous during all year. This is not a normal physiological rhythm for the laboratory-bred stock animals. If this artificial condition persists for a long time, it may occur that animals try to defend their metabolic status by inactivating receptors or reducing their steroid hormone levels. In this condition, the detection of receptors or hormones is sticky by IHC, ELISA or other analytical methods. The conclusion will be an artifact about the presence and distribution of steroid receptor or hormone in different tissue of snail.

The fact is that vertebrate-like steroid hormones undoubtedly can be detected in molluscs. Whether they are synthesized and performed physiologically, relevant functions taken up from the environment is firmly not established yet. The solution to mention problems are for scientists to apply more robust experimental designs and animals in sufficient conditions [95].

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Author details

Zsolt Pirger*, Zita Zrinyi, Gábor Maász, Éva Molnár and Tibor Kiss

*Address all correspondence to: pirger.zsolt@okologia.mta.hu

MTA-ÖK BLI NAP_B Adaptive Neuroethology, Department of Experimental Zoology, Balaton Limnological Institute, MTA Centre for Ecological Research, Tihany, Hungary

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