In the course of our research project we have investigated the organization, functional anatomy and physiological role of different sensory systems of aquatic and terrestrial invertebrates, molluscan species. The series of the studies involved the following items: I. Sensory systems and their possible role in sensing and processing environmental cues; II. Inter- and intracellular events in processing odor information and feeding; III. Effect of human disruptors on development and behavior.

## I. Peripheral elements of the nervous system of the biofouling zebra mussel, *Dreissena*, playing a possible role in its successful invasive behavior

### I/1. Early sensory elements in the developing nervous system of *Dreissena*

Zebra mussel, Dreissena sp., has been the ruling bivalve species in freshwater lakes, including Lake Balaton, within decades, raising the question what might be the basis of its successful invasion. One possibility is to look for the nervous system commanding both early and adult adaptive behaviors needed for optimal responses to environment challenges. Based on it, we have investigated the embryonic development of the nervous system in early larval stages of Dreissena, with special attention to their signal molecules (5-HT, FMRFamide [Fa]) content. Fluorescence immunohistochemistry revealed that the first apical 5-HT-immunoreactive (IR) sensory cells appeared already as early as in 16-18 hours old post-fertilization free living trochophores, followed by additional 5-HT-IR interneurons in the apical organ (AO), as well as by 5-HT-IR a posterior and the stomach cells sending axon processes to the AO from 32-40 hours post-fertilization (hpf) veliger larvae **(Figs. 1)**.

Fa-IR sensory neurons appear first from 32 hpf stage in the AO, followed later by additional neurons appearing in the anterior lateral and posterior lateral as well as posterior regions, all being interconnected with AO **(Figs. 2)**. It seems that three 5-HTergic and Faergic sensory centers, respectively, exist during early embryogenesis of Dreissena, from which the AO may function as a simple "brain", processing sensory stimuli arriving from both the external (anterior and posterior sensory cells) and internal (vegetative, stomach neuron) world.

The role of 5-HT in swimming was proven by demonstrating that the precursor 5-HTP evoked an elevated swimming activity of 48 hpf veliger larvae, whereas blocking the 5-HT synthesis by pCPA resulted in a reduced swimming activity.

As a possible (negative) environmental effect, we have tested the influence of increased salinity on the 5-HT and Fa immunoreactivity, respectively, in identified neuronal structures of the nervous system, as well as on the swimming activity of 48 hpf veligers (**Fig. 3**). After 24 hours incubation in Balaton-water containing 2 g/L (2‰) NaCl, both 5-HT-IR and Fa-IR neurons **(Fig. 3A, A')** revealed increased fluorescence intensity **(Fig 3B, B')**. Quantification of the relative fluorescence intensity showed that the larval neuronal elements investigated altered significantly (20-150%) their signal molecule content in response to increased salinity (**Fig. 3C, C'**). Increased salinity also induced elevated swimming activity of the veliger larvae.

Future perspectives and tasks may and will involve the manipulation of larval behavior by different (allele) chemical cues of aquatic plant origin, to obtain view of how zebra mussel are capable of resisting to repellent effects.

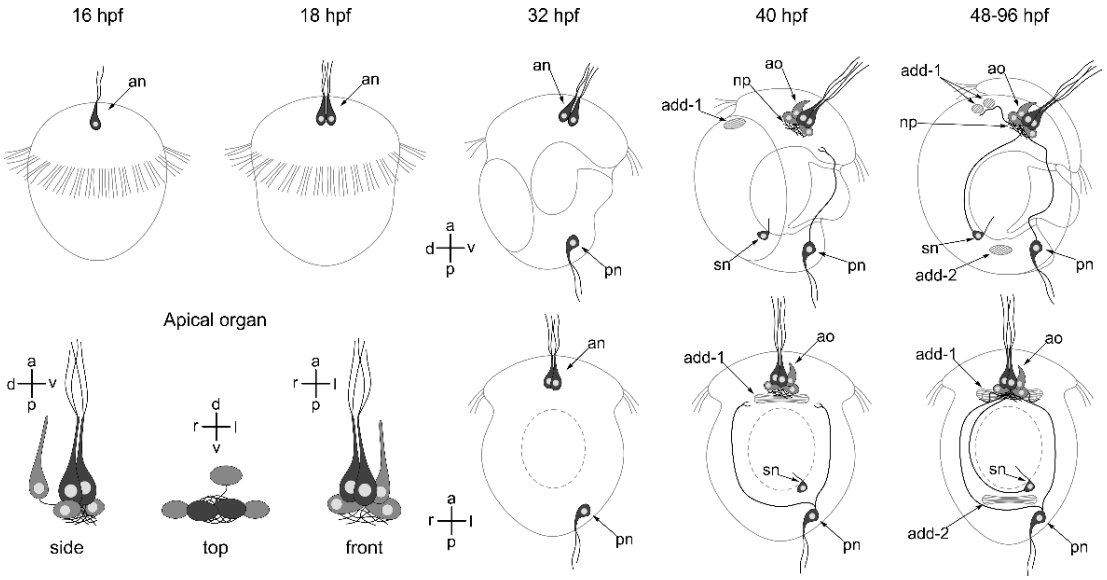



**Fig. 1.** Early larval development of the 5-HT-IR sensory system in Dreissena. an – anterior sensory cells, ao – apical organ, np – neuropil, pn – posterior neurons, sn – stomach neuron, add – adductor muscle

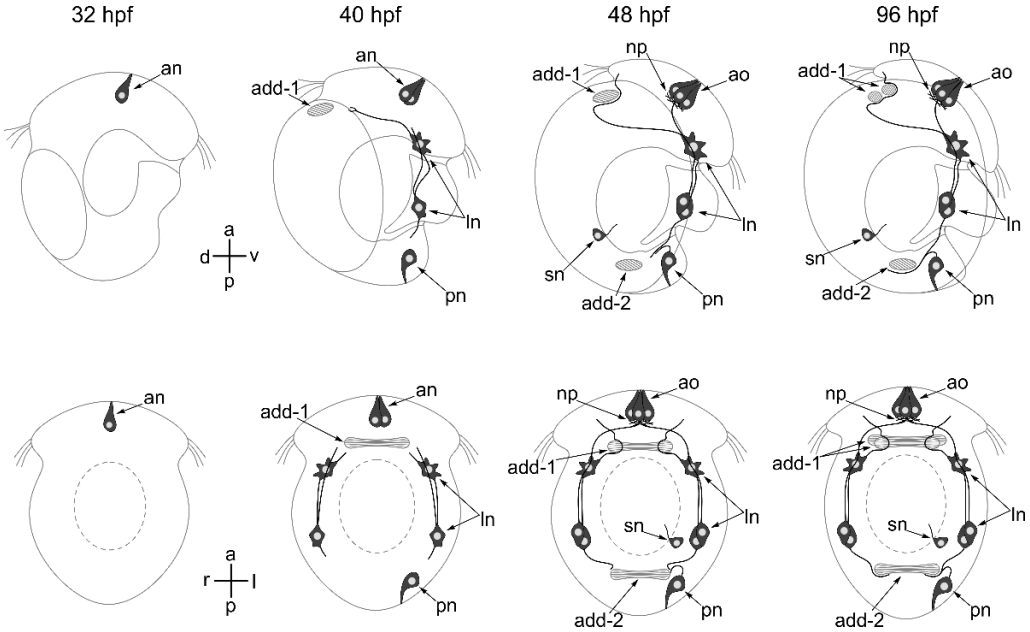



**Fig. 2.** Early larval development of the FMRFa-IR nervous system in Dreissena. an – anterior sensory cells, ao – apical organ, np – neuropil, pn – posterior neurons, ln – lateral neurons, sn – stomach neuron, add – adductor muscle

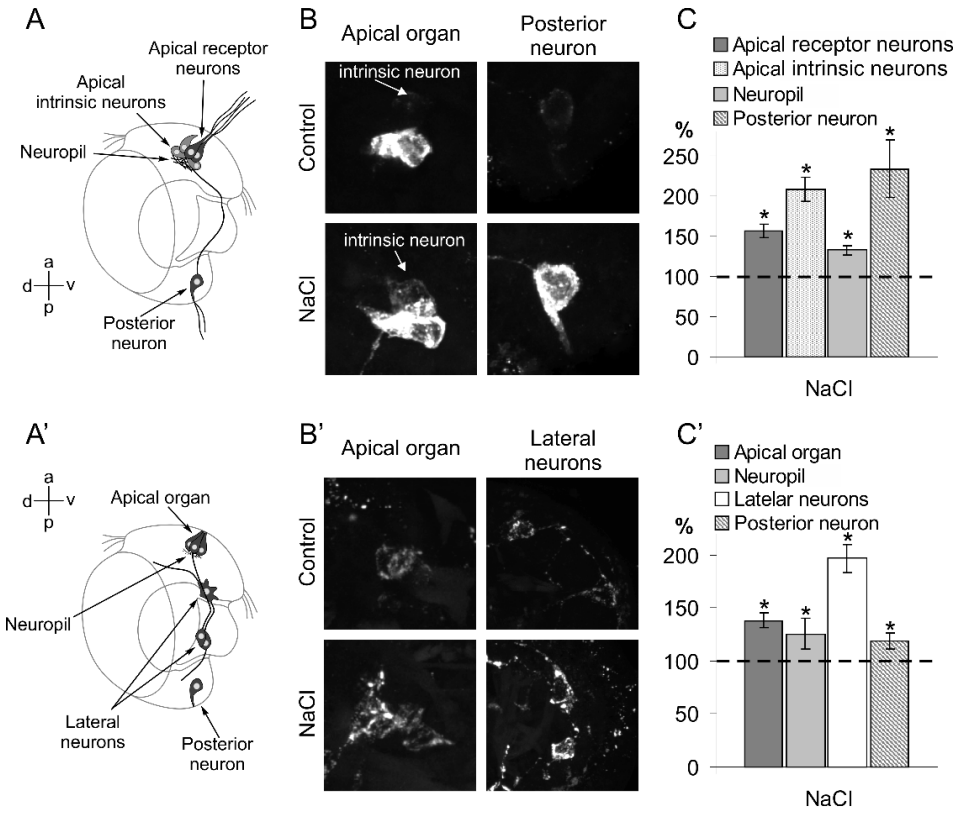



**Fig. 3.** Effect of increased salinity on the fluorescence intensity (B, B', C C') of 5-HT-IR (A) and FMRFa-IR (A') cells.

## I/2. Innervation of the byssus retractor muscle and foot of *Dreissena*

Zebra mussels (*Dreissena* sp.) are strongly attached to hard surfaces via the bysuss, an organ containing numerous proteinaceous threads **(Fig. 4A)**, which originates from the foot and are controlled by the byssus retractor muscle (BRM). By this organ the bivalves are capable of resisting to even aggressive physical effects, such as strong waves, and so remaining at their optimal living territory. To reveal the innervation characteristics of the BRM, we have performed fluorescence immunohistochemistry (IHC) for the visualization of choline-acetyltransferase (ChAT; acetylcholine is the excitatory transmitter), and the neuromodulators, serotonin (5-HT) and two endogenous molluscan neuropeptides, FMRFamide (Fa) and Mytilus inhibitor peptide (MIP). Networks established by 5-HT-IR and Fa-IR fibers were observed around the BRM. From the stem part of the BRM finger-like processes extended into the internal part of the foot, along which varicose 5-HT-IR **(Fig. 4A)** and Fa-IR fibers first surrounded the stem, and then ran parallel very close to the finger-like extensions, suggesting an overall innervation of the BRM. By double immunostaining with anti-Fa and α-actinin a more precise neuromuscular relationship could be demonstrated **(Fig. 4B)**. ChAT-IR fibers ran also along the finger-like protrusions, supporting the role of acetylcholine in the muscle contraction. Our findings refers to a complex cholinergic, aminergic and peptidergic regulation of the BRM function.

Ultrastructural investigations were also performed to reveal the fine structural organization of the BRM and its innervating elements. It was established that: i) the inner surface of the BRM system was covered by an epithelial layer densely supplied by cilia; ii) smooth muscle fibers were seen to intrude deeply between the epithelial cells; iii) smooth muscle fibers located in deeper regions beneath the epithelia layer were accompanied by small axon bundles; iv) both unlabeled varicosities and small and large size Fa-IR profiles (**Fig. 4D**) were found to contact muscles fibers or sarcoplasmic processes. The organization and innervation pattern suggest that

both the muscle fibers and the ciliated epithelium layer are equally involved in protruding the byssus stem, whereas the innervated muscles fibers ensure the long-lasting attachment to the surface by the protruded threads.

Battonyai I., Elekes K. (2019) Localization of neurotransmitters responsible for the regulation of byssus retractor muscle of the biofouling mussel, *Dreissena polymorpha*. An immunohistchemical study. (Manuscript)

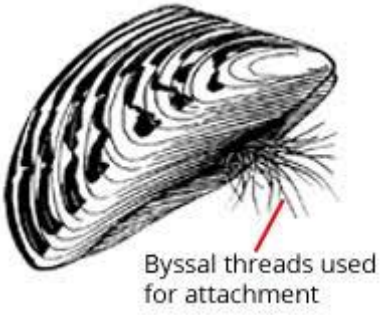
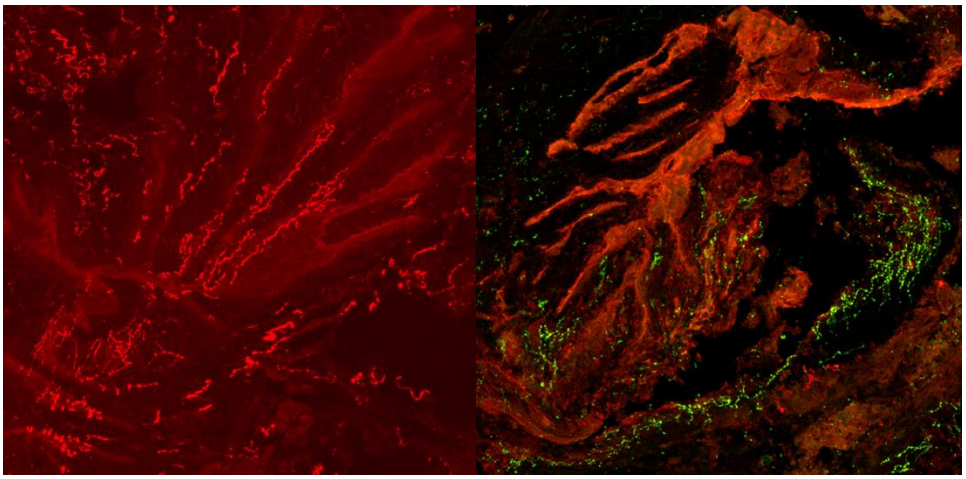



**Fig. 4A** Drawing of a zebra mussel with protruded byssal threads



**B** **C**

**Fig. 4B** 5-HT-IR innervating fibers (red) innervating the BRM. **Fig. 4C** Actinin-IR BRM (red) surrounded by Fa-IR fibers (green)

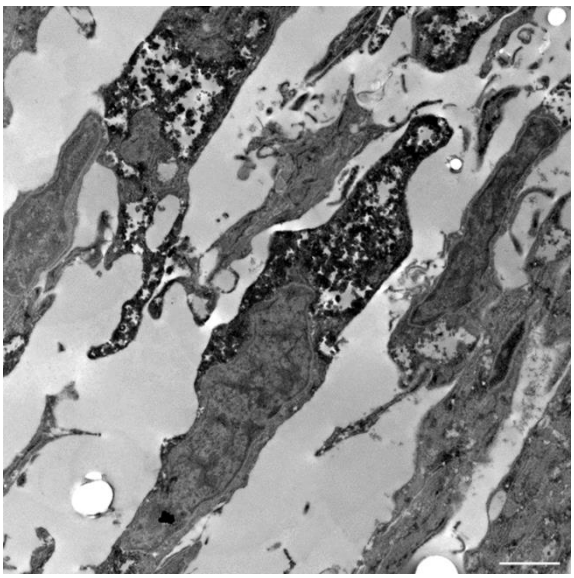


**Fig. 4D** TEM view of the BRM showing large size Fa-IR axon profiles contacting muscle fibers

## II. Innervation of peripheral regions in the pond snail, *Lymnaea stagnalis*, with special attention to the relationship of sensory and efferent elements

### II/1 Signaling molecules in the sensory-efferent system

The peripheral nervous system plays a decisive role in the neural control of behavior in the pond snail (*Lymnaea stagnalis*). Although there are information on a number of sensory elements, we have little knowledge on the complex organization of the peripheral sensory-efferent system, especially regarding the relationship between the different signaling systems. To obtain data on this point, we have applied single and double-labeling IHC on the lips, tentacles, and foot of adult *Lymnaea*, using antibodies raised against serotonin (5-HT), histamine (HA), tyrosine-hydroxylase (TH), glutamate (Glu), and the endogenous molluscan neuropeptides FMRFa (Fa) and Mytilus inhibitory peptide (MIP). It was demonstrated that HA-IR, TH-IR, Glu-IR-, Fa-IR and MIP-IR bipolar sensory cells were present throughout in the sensory epithelium of the three peripheral regions. The exclusively extrinsic (CNS) 5-HT-IR innervation was prominent, forming both sub-epithelial and deeper (muscle layer) networks. In addition, dense Fa-IR, MIP-IR and loosen HA-IR and Glu-IR sub-epithelial networks could also be observed Based on it, both complex interaction between and simultaneous parallel action of the different signal molecules in the *Lymnaea* periphery are assumed (**Fig. 5**), in which the sub-epithelial 5-HTergic network seems to be the decisive element of central origin, modulating in loco the peripheral processing of sensory input.

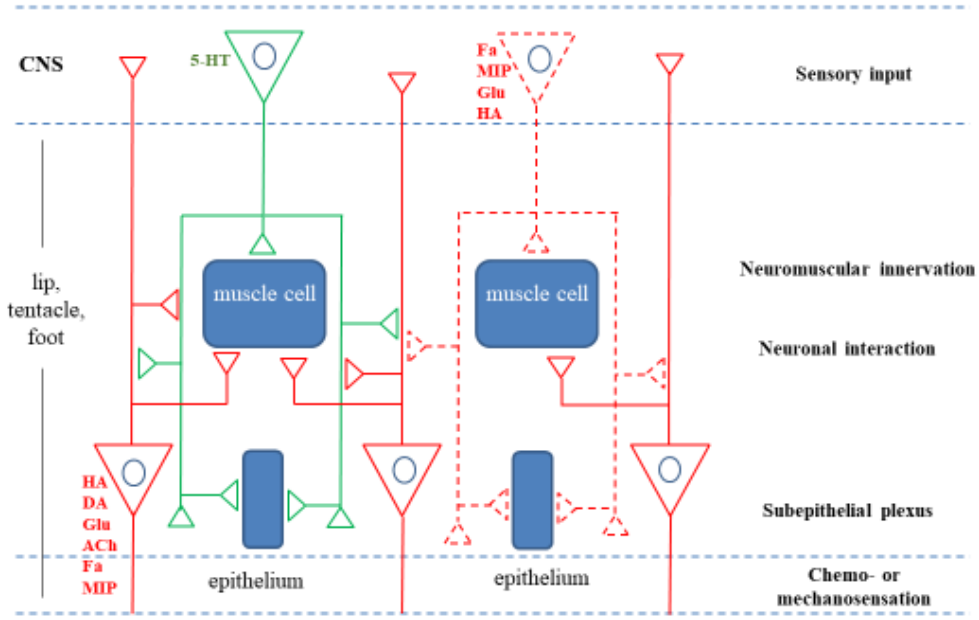



**Fig. 5** Schematic representation of the complex organization of the different signaling systems in the periphery of *Lymnaea*.

### II/2 Ultrastructure of the sensory regions

According to ultrastructural (TEM) studies, the sensory epithelium of the lip and foot consisted of a densely arranged layer of ciliated or un-ciliated sensory cells. The cells were highly elongated, contained a moderately electron dense cytoplasm with numerous mitochondria, rER elements and free ribosomes. The sensory epithelial layer was separated by a wide extracellular space from the sub-epithelial region, containing sensory axon bundles and gland cells. The sub-

epithelial region was dominated by the presence of smooth muscle fibers, among which smaller and larger axon bundles were located, which contained profiles with large (80-120 nm) electron dense granules. In addition, bundles of axon profiles of small diameter also occurred, possibly representing sensory fibers. Neuro-muscular contacts characterized by long, parallel arranged unspecialized membrane segments were found regularly, whereas neuro-glandular contacts could occasionally be observed. Correlative light- and electron microscopic studies on the visualization of intercellular contacts of 5-HT-IR and Fa-IR elements are in progress.

**II/3 Determination of different signal molecules in the peripheral organs**

HPLC-MS measurements were carried out in order to identify and quantify the concentration of different signal molecules (5-HT, dopamine, GABA, HA, Glu, acetylcholine) in the foot, lip and tentacles. All the signal molecules assayed were detected in the peripheral organs, although, with strongly different concentrations (ng/mg) as follows: Glu (lip – 101,667; tentacles – 165,369; foot – 110,136) > 5-HT (lip – 42,683, foot – 21,443, tentacles - 51,222) > acetylcholine (lip, - 17,851, foot – 5,740 , tentacles - 4,119) > dopamine (lip - 5,568, foot - 6,095, tentacles - 5, 971) > GABA (lip - 2,149, foot - 0,839, 1,702 – tentacles) > HA (0,193 – lip, foot - 0, 082, tentacles - 0,297).

Horváth R., Battonyai I., Maász G., Schmidt J., Fekete N. Zs., Elekes K. (2019) Organization of peripheral sensory-efferent systems in the pond snail *(Lymnaea stagnalis)*. A light- and electron microscopic immunocytochemical study. (Manuscript ready for submission)

**III. The nitrogen oxidergic system in gastropods**

**III/1 NO regulated intracellular messengers in the olfactory lobe**

Olfaction, a chemosensory modality, plays a pivotal role in the orientation and behavior of invertebrates. The central olfactory processing unit in terrestrial stylomatophoran snails is the procerebrum (PC), which contains NO synthesizing interneurons, whose oscillatory currents are believed to be the base of odor evoked memory formation. However, in this model the up- and downstream events of molecular cascades that trigger and follow NO release, respectively, have not yet been studied. Therefore, we have investigated the molecular events related to the release of nitrogen oxide (NO) in the PC of the snail, *Helix pomatia*, with special attention to the cascades involved in NO release. Immunocytochemistry and flow cytometry studies performed on isolated PC neural perikarya revealed cell populations with discrete DAF-2 fluorescence, indicating the release of different amounts of NO **(Fig. 6).** Glutamate (Glu) increased the intensity of DAF-2 fluorescence and the number of DAF-2 positive non-bursting interneurons through a mechanism likely to involve an NMDA-like receptor. Similarly to Glu, NO activation induced an increase in intracellular cGMP level through activation of soluble guanylyl cyclase. Immunohistochemical localization of proteins possessing the phosphorylated target sequence of AGC family kinases (RXXS/T-P), among them protein kinase A (RRXS/T-P), showed striking similarities to the distribution of NOS/cGMP. Activators of cyclic nucleotide synthesis increased the AGC-kinase-dependent phosphorylation of discrete proteins with 28, 45, and 55kDamw. Exposure of snails to an attractive odorant induced hyper-phosphorylation of the 28kDa protein, and increased levels of cGMP synthesis. Protein S-nitrosylation and intercellular activation of protein kinase G were also suggested as alternative components of NO signaling in the snail PC. The present results indicate an important role for the procerebral NO/cGMP/PKA signaling pathways in the regulation of olfactory (food-finding) behavior.

## III/2 Ultrastructural localization of NADPH diaphorase and nitric oxide synthase in the neuropil of the snail CNS

Comparative studies on the nervous system revealed that NO retains its function through the evolution. In vertebrates NO can act in different ways: released either solely or as a co-transmitter from presynaptic or postsynaptic sites, after which it spreads as a volumetric signal or targets synaptic proteins. In invertebrates, however, the possible sites of NO release have not yet been revealed. Therefore, we have investigated the subcellular distribution of the NO synthase (NOS) in the central nervous system (CNS) of two gastropod species, the terrestrial snail, Helix pomatia and the pond snail, Lymnaea stagnalis. For the visualization of NOS, NADPH-diaphorase histochemistry and an immunohistochemical procedure using a universal anti-NOS antibody were applied. At light microscopic level, both techniques labeled identical structures in sensory tracts ramifying in the neuropil and cell bodies of the Helix and Lymnaea CNS. At ultrastructural level both NADPH-d reactive and NOS-immunoreactive (IR) materials were localized on the nuclear envelope and membrane segments of the rough and smooth endoplasmic reticulum, as well as along the and axolemma and cell body membrane of the labeled perikarya. NADPH-d reactive and NOS-IR varicosities were connected to neighboring neurons with both unspecialized and specialized synaptic contacts. In the varicosities, the majority of the NADPH-d reactive/NOS-IR membrane segments were detected in round and pleomorph agranular vesicles of small size (50-200 nm). However, altogether only a small portion (16%) of the vesicles displayed the NADPH-d or NOS labeling. No evidence for the postsynaptic location of NOS was found. Our results suggest an identical localization of NADPH-diaphorase and NOS in the snail nervous system. In contrast to vertebrates, however, NO seems to act exclusively in an anterograde way, possibly released from membrane segments of the presynaptic transmitter vesicle surface. Based on the subcellular distribution of NOS, NO could be both a volume and a synaptic mediator, and, in addition, NO may function as a co-transmitter.

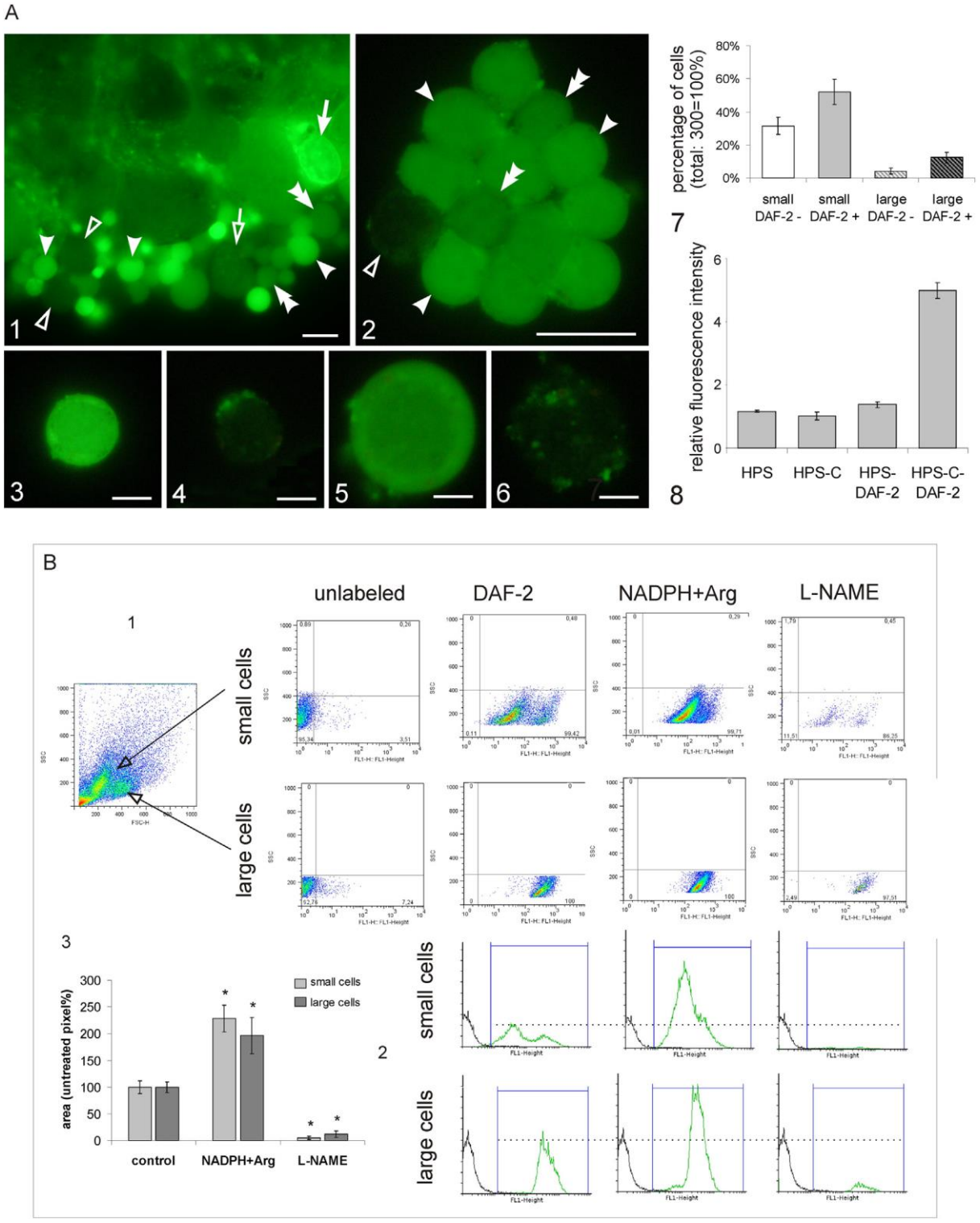



**Fig. 6A** DAF-2-DA labeled NO producing PC neurons shown in whole-mount preparation (A1) and as isolated cells (A2– 6). **B** Cell flows, DAF-2 fluorescence of cells and its change after incubation with NOS co-factor mix (1 μM NADPH + arginine) or 100 μM L-NAME.

## IV. Serotonergic regulation of the buccal (feeding) rhythm of the pond snail, *Lymnaea stagnalis*

Hatching is an important phase of the development of pulmonate gastropods followed by the adult-like extracapsular foraging life. Right before hatching the juveniles start to display a rhythmic radula movement (adult-like feeding activity), executed by the buccal complex which consists of the buccal musculature (mass) and a pair of the buccal ganglia. In order to have a detailed insight into this process, we investigated the 5-HTergic regulation of the buccal (feeding) rhythm in 100% stage embryos of the pond snail, Lymnaea stagnalis, applying quantitative immunohistochemistry combined with either stimulating (by the 5-HT precursor 5-hydroxytryptophan, 5-HTP) or inhibiting (by the 5-HT synthesis blocker para-chlorophenylalanine, pCPA) 5-HT synthesis. Correspondingly, significant changes of the fluorescence intensity could be detected both in the cerebral and the buccal complex. HPLC-MS assay demonstrated that 5-HTP increased, meanwhile pCPA decreased the 5-HT content both of the central ganglia and the buccal complex. As to the feeding activity, 5-HTP induced a slight (20%) increase, whereas the pCPA resulted in a slight decrease (20%) of the radula protrusion frequency. Inhibition of 5-HT re-uptake by clomipramine reduced the frequency by 75%. The results prove the role of both central and peripheral 5-HTergic processes in the regulation of feeding activity. Application of specific receptor agonists and antagonists revealed that activation of a 5-HT1-like receptor depressed the feeding activity, meanwhile activation of a 5-HT6,7-like receptor enhanced it **(Fig. 7)**. Saturation binding plot of [3H]-5-HT to receptor and binding experiments performed on membrane pellets prepared from the buccal mass indicated the presence of a 5-HT6-like receptor positively coupled to cAMP. The results indicate that 5-HT influences the buccal (feeding) rhythmic activity in two ways: an inhibitory action is probably exerted via 5-HT1-like receptors, while an excitatory action is realized through 5-HT6,7-like receptors.

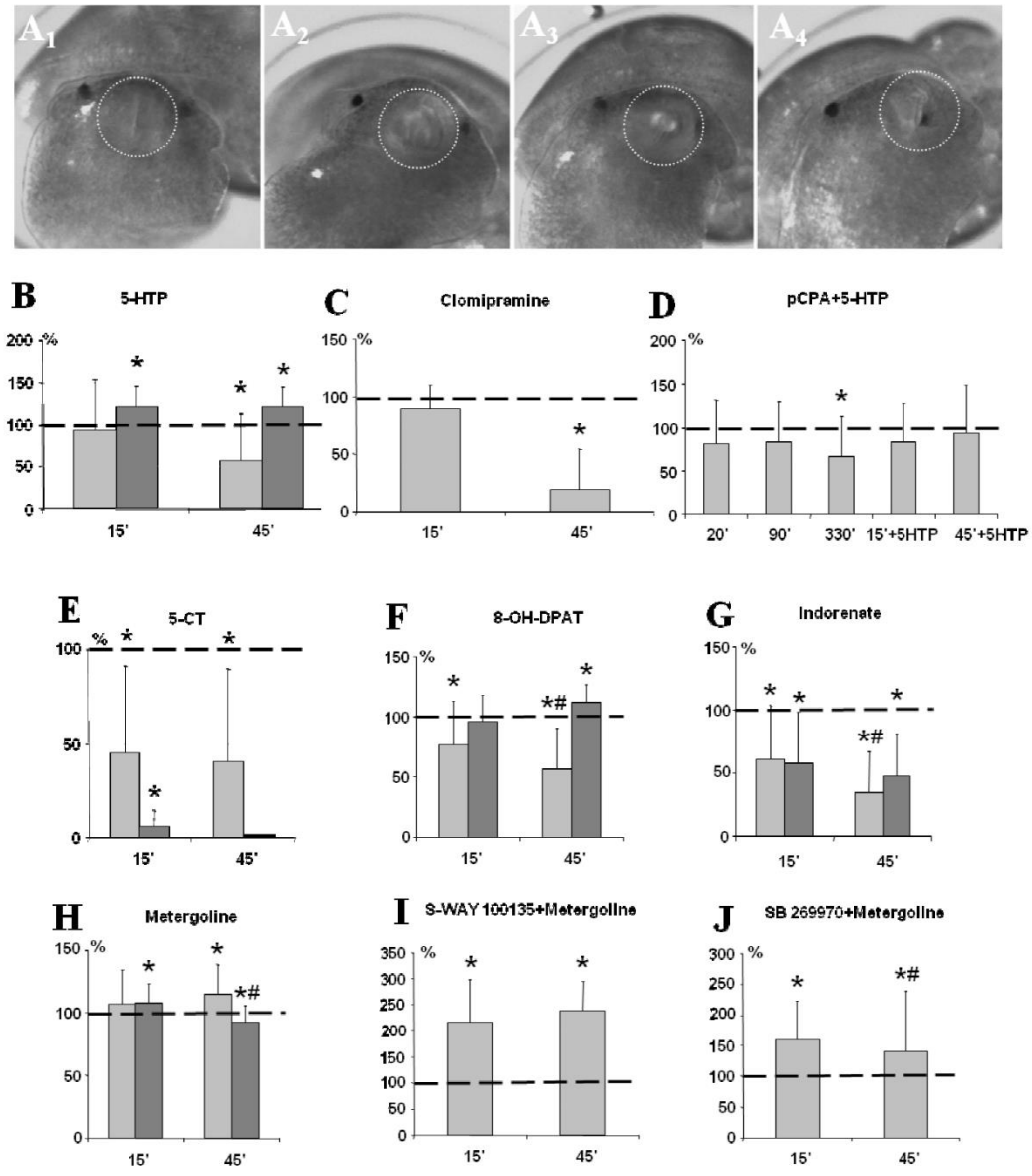



**Fig. 7.** 5-HTergic regulation of feeding activity of Lymnaea. **A** Different phases of feeding activity (encircled, A1: mouth closed; A2: radula protraction, A3: rasping; A4: radula retraction). **B** Pharmacological manipulation of the 5-HTergic regulation of radula protrusion. Light grey: lower, dark gray: higher drug concentration.

## V. Effect of environmental chemical factors on aquatic invertebrates

### V/1. Effect of allelochemicals on the behavior and neuronal regulation of feeding of the pond snail, *Lymnaea stagnalis*

Aquatic animals are constantly exposed to harmful or aversive (repellent) chemical substances, such as e.g. allelochemicals. One of them is tannic acid (TA) which is a polyphenol, belongs to the galloylglucose family, and is produced by different plant species as a secondary metabolite. Our aim was to clarify the possible effects of this compound on the locomotion and feeding of *Lymnaea stagnalis*.

### V/1/1. Effect of TA on the locomotion

Following the application of different TA concentrations (10 μM, 100 μM), the locomotion of the animals was strongly influenced, showing a time and concentration dependent tendency. TA evoked gradually the prevalence of passive movement forms, reflected either by sticking or floating, meanwhile active forms as sliding and swimming became less and less typical.

### V/1/2 Effect of TA on the feeding activity

Feeding tests with intact animals showed that although the presence of 10 or 100 μM TA did not prevent the snails to respond to 0.1 M sucrose, 100 μM TA significantly increased the time duration measured between food (sucrose) application and the start of feeding activity (radula protrusion) and also decreased the frequency of the feeding cycles **(Fig. 8)**.

The results clearly demonstrate the negative (inhibitory) effect of TA on both the locomotion and the feeding behavior of *Lymnaea*.

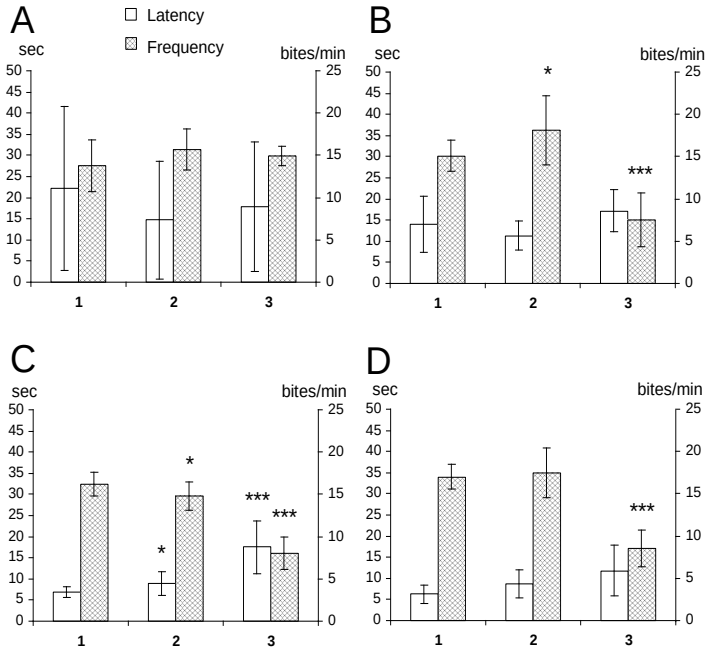



**Fig. 8.** Feeding tests performed on snails following the application of 100 mM sucrose (control) and after adding TA. **A** In control groups feeding latency and feeding rate is similar. **B** Increased feeding rate in group 2 (10 μM TA) and decreased feeding frequency in group 3 (100 μM TA), compared to control (group 1). **C** Repeated tests next day (1: sucrose; 2: sucrose + 10 μM TA; 3: sucrose + 100 μM TA) show significantly longer latency and lower feeding rate in both treated groups (2, 3). **D** All the groups (1, 2, 3) tested after 48 h in sucrose only. Left vertical axis: latency in seconds, right vertical axis: feeding frequencies expressed as bite/min.

**V/1/3 Effect of TA on the feeding regulatory system**

After the application to the lip/mouth region, the effect of TA was analyzed in semi-intact preparation at the level of central neurons which are responsible for the regulation of the feeding rhythm. In addition, the effect of direct application to the key modulatory member of the central feeding network, the 5-HTergic giant cell (CGC), was also investigated. Electrophysiological recordings from identified neurons of the feeding network in the buccal ganglion showed a decreasing spontaneous activity and also reduced responses evoked by feeding stimuli of 100 μM sucrose applied to the lip area **(Fig. 9)**. In the cyclic activity of feeding pattern some central input arriving from the CPG network also seemed to be changed. However, the frequency of the DA evoked feeding rhythm decreased, suggesting that the CPG network is not directly inhibited by TA. Also, neither the activity (spontaneous firing), nor the electrophysiological parameters (membrane potential level, amplitude, action potentials) of the 5-HTergic CGC were altered in the presence of TA. The results suggest that the TA induced longer lasting feeding inhibition is most likely due to the peripheral impairment of the sensory pathways of the feeding system in *Lymnaea*.

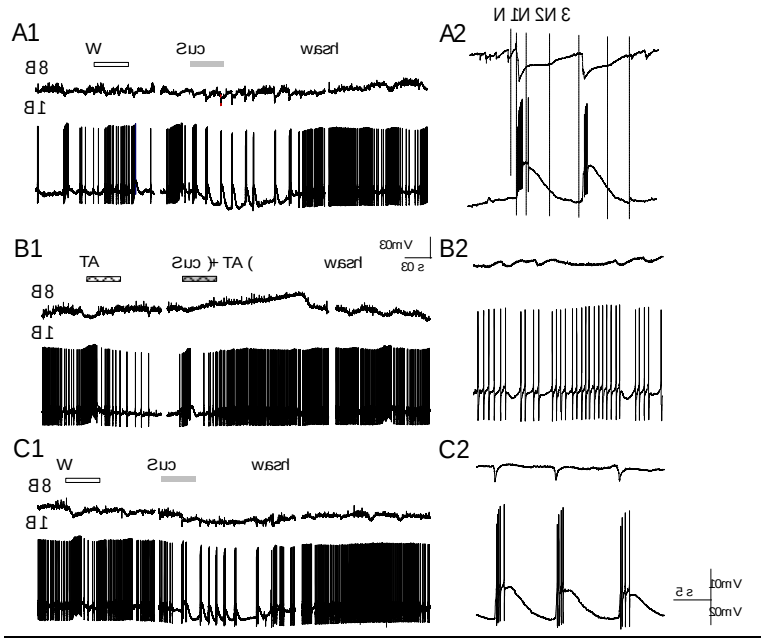



**Fig. 9.** TA prevents feeding response to sucrose. **A1** Simultaneous recording from two buccal motoneurons (B8, B1) in Balaton water (W), followed by the application of 100 mM sucrose (Suc) to the lip. **A2** Sucrose-evoked rhythmic activity is seen as series of inhibitory inputs on B8 neuron and depolarization with action potentials on B1 neuron, respectively. N1, N2, N3: phases of fictive feeding. **B** Application of 100 μM TA did not change the spontaneous activity. **B1** Sucrose added to TA solution (Suc +TA) fails to evoke the feeding rhythm, **B2** Rhythmic synaptic inputs are not visible in B8 or B1 neurons. **C** After 20-min washing, the lip sucrose-evoked intracellular response is recovered (**C1**) and the cyclic synaptic inputs re-appear in the intracellular activities of feeding neurons. (**C2**).

**V/1/4 Effect of TA on the *Lymnaea* sensory system**

Based on the above described behavioral and electrophysiological results, we have started qualitative and quantitative immunohistochemical as well as biochemical (HPLC-MS) measurements following TA administration (10 or 100 μM for 60 or 120 min). Four experimental groups (each consisting of 3 animals) were used as follows. Following the treatments the three peripheral organs (lip, tenctacle and foot) were processed either for 5-HT,

TH, histamine (HA), glutamate (Glu) and ChAT IHC, respectively, or for 5-HT, dopamine, GABA, HA, Glu and acetylcholine HPLC-MS assay. Preliminary observations indicated reduced intensity of immunolabeling in the epithelial sensory elements whereas the sub-epithelial plexi seemed to be unchanged following both 60 and 120 min100 μM TA treatment. HPLC-MS assays are in progress.

**V/2/1 Effects of human disruptors on the embryogenesis and embryonic behavior of the pond snail, *Lymnaea stagnalis***

Recently, steroidal estrogen and progestogen compounds have become part of the most studied pharmaceutical pollutants in freshwater ecosystems. However, only a few studies explored the effect of progestogen treatment in a mixture to reveal how non-target species respond to these contaminants. In our study, specimens of the pond snail, *Lymnaea stagnalis*, were exposed to a mixture of four progestogens (progesterone, levonorgestrel, drospirenone, and gestodene) in 10 ng/L concentration for 3 weeks. Effects both at physiological and cellular/molecular level were analyzed using ELISA technique, stereomicroscopy combined with time lapse software, and capillary micro-sampling combined with mass spectrometry. The treatment of adult *Lymnaea* specimens resulted in reduced egg production and low quality egg mass in the first week, compared to the control. From the second week, the egg production and the quality of egg mass were similar both in the treated and control groups. At the end of the third week, the egg production and the vitellogenin-like protein content of the hepatopancreas were significantly elevated in the treated animals. At the cellular level, accelerated cell proliferation was observed during early embryogenesis in the treated group. The investigation of metabolomic changes revealed significantly elevated hexose utilization in the single-cell zygote cytoplasm and enhanced adenylate energy charge in the egg albumen **(Fig. 10)**. These observations suggest that treated snails provided more hexose in the eggs in order to improve offspring viability. Our results contributes to the knowledge of the physiological effect of progestogen mixture at environmentally relevant dose on non-target aquatic species, and also offer the possibility to analyze the neuro-developmental aspects of the progestogen effects.

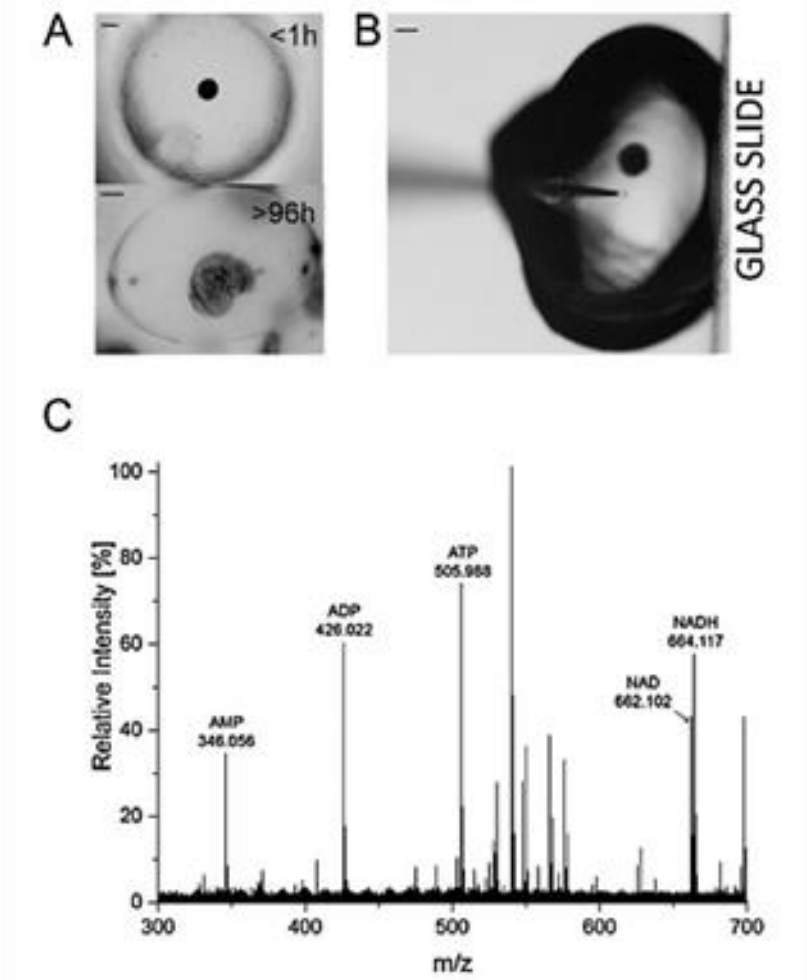



**Fig. 10.** Microsampling for metabolomics analysis of albumen **A** A 1-h (upper) and a 96-h (lower) old egg. **B** The capillary micro-sampling of albumen from a 1-h old egg. Arrow - capillary ending. Scale bars: 100μm. **C** - A representative mass spectrum of albumen taken in 300–700 m/z detection range, presenting the highlighted molecules of AEC and redox state.

**V/2/2 Effects of human disruptors on the feeding behavior and its neuronal background in *Lymnaea***

We also investigated the adaptive changes induced by a mixture of progestogens (progesterone [PRG], drospirenone [DRO], levonorgestrel [LNG], gestaden [GES]) found in commercially available oral contraceptives and occurring in natural aquatic environments, like Lake Balaton. *Lymnaea* specimens were injected by 1 or 100 ng/L progestogen mixture and their feeding behavior was compared with that of the control, followed by intracellular electrophysiological recordings from different components of the identified members of the neuronal networks responsible for controlling feeding. At behavioral level, snails treated with the progestogen mixture displayed a reduced feeding (rasping) activity to sucrose used as feeding stimulus, compared to control animals. At cellular level, the hormone treatments evoked decreased firing frequency of the key feeding modulatory interneuron, the cerebral giant cell (CGC) by increasing the amplitude of $Ca^{2+}$ currents. This effect seemed to be cell type specific, since in the case of the respiratory CPG cell (right pedal-dorsal cell, RPeD1) a similar change could not be observed. On the other hand, feeding (B1-B3) motoneurons located in the buccal ganglia also displayed a decreased firing activity during fictive feeding stimulus, indicating a reduced responsiveness to the external sucrose application.

We also investigated the possible eco-physiological effect of progestogens on embryos and adult individuals of pond snail (Lymnaea stagnalis) and in young adults (5-6 days) of the water flea (Cladocera, Daphnia magna), regarding possible changes caused at the behavioral level and the growth intensity. Chronic treatments with 1, 10, 100 or 500 ng/l mixture of progestogens (progesterone, levonorgestrel, drospirenone, gestodene) were applied, modelling the environmental effects of pilot areas under controlled laboratory conditions It could be established that, in case of late (90%) Lymnaea embryos, the rate of heartbeats did not change significantly, but the number of radula movement (feeding activity) significantly increased and the embryonic growth was also accelerated in the presence of already 1 ng/l progestogen mixture. In adult snails, the feeding activity also significantly increased compared to the control, while the locomotion decreased. In case of Daphnia, the rate of growth after hatching changed, depending on the concentration of the hormone treatment applied. Also, following hormone exposure, the number of young females bearing eggs did not change significantly compared to the control group, but the number of eggs in the female water fleas was reduced, depending on the applied concentration of mixture in a hormatic way. The number of neonates correlated with the increase of number of eggs. The results show that contaminants of human oral contraceptives and their active degradation products may represent a true negative challenge to the members of aquatic ecosystems.

## VII. Conclusions, future aspects and possible ways of further investigations

Based on the brief summaries of our abovementioned investigations and obtained results, we can conclude that invertebrates, this time molluscan models species and their nervous system, is and will remain indispensable targets to know more about basic processes detecting, interpreting and responding to chemical cues of environmental origin emitted different living organism or as human polluting agents. The possible ways of further studies may include the analysis of different environmental chemical impacts: i) at the level of intracellular molecular cascades (see para II, III), ii) at the level of sensory information tracing along intramembranous molecular (receptor) and intercellular connection systems (see paras I, II, III, IV), and finally, and perhaps most importantly, iii) at cellular and organism developmental levels (paras I, II, V) which may enlighten the “birth” of the organization of regulatory and signaling systems which are responsible for early interpretation of external messages that may enable the living organism for proper responses and the optimal preparation for the adult life.