Neuronal Transcriptome Analysis of a Widely Recognised Molluscan Model Organism Highlights the Absence of Key Proteins Involved in the De Novo Synthesis and Receptor-Mediation of Sex Steroids in Vertebrates

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NEURONAL TRANSCRIPTOME ANALYSIS OF A WIDELY RECOGNISED MOLLUSCAN MODEL ORGANISM HIGHLIGHTS THE ABSENCE OF KEY PROTEINS INVOLVED IN THE DE NOVO SYNTHESIS AND RECEPTOR-MEDIATION OF SEX STEROIDS IN VERTEBRATES

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ABSTRACT

Over the last ten years, the interpretation of the presence of vertebrate sex steroids in molluscs has changed dramatically. Evidence has been accumulating that CYP11A and CYP19A genes (encoding cholesterol side-chain cleavage enzyme and aromatase), that are crucial for the biosynthesis of sex steroids in vertebrates, as well as key functional sex steroid receptors, are missing in molluscan genomes. To provide further evidence, we sequenced the whole transcriptome of the central nervous system of the great pond snail (*Lymnaea stagnalis*) and screened it for sequences homologous to those used in the generally accepted vertebrate sex steroidogenesis pathway as well as the known sex steroid receptor-related genes (such as CYP11A, CYP19A, 3β-HSD, nPR, and nAR). Our screening confirmed the absence of several key sequences that are essential to accomplish a full sex steroid biosynthesis pathway similar to that of vertebrates. There was also no evidence for nuclear sex steroid receptors. Our findings support the contention that molluscan endocrinology differs from the well-characterized vertebrate endocrine system.

Key words: sex steroidogenesis, sex steroid receptors, progesterone, testosterone, estrogen, mollusc, mollusk, the great pond snail, *Lymnaea stagnalis*.

INTRODUCTION

There is a continuing debate about the functionality of sex steroids in molluscan reproductive processes and their (neuro)endocrine systems. Although vertebrate-like sex steroids are present in molluscs, it has been argued that they are so readily absorbed from their environment that their presence is not necessarily evidence of endogenous synthesis (Scott, 2018). Indeed, emerging evidence suggests that molluscan endocrinology differs in many ways from the well-characterized vertebrate endocrine system, especially in the ability to biosynthesize sex steroids (reviewed by Fodor et al., 2020a; Scott, 2012, 2013, 2018). There are two generic types of chemical reaction involved in the formation of vertebrate steroids. The first, relatively simple one, involves the insertion or removal of two hydrogen atoms (i.e., oxidoreductase reactions). This type of reaction occurs in all living organisms and with all sorts of compounds (i.e., not just steroids). Evidence for the occurrence of such oxidoreductase reactions in molluscs is strong – for example, conversion of pregnenolone to progesterone by *Octopus vulgaris* (Di Cristo et al., 2010) and conversion of testosterone to 5α-dihydrotestosterone by *Mytilus edulis* (Schwarz et al., 2017). However, the evidence that these reactions form part of a steroid biosynthetic pathway equivalent to that of vertebrates is circumstantial. The second, more complex type of reaction involves oxygen atom insertions, carried out by enzymes that belong to the Cytochrome P450 family (CYP); but there is as of yet no conclusive evidence that these reactions occur in molluscs since homologous genes to two out of three of these enzymes have not yet been found in molluscs (reviewed by Fodor et al., 2020a; Scott, 2012).

In a previous study, reference was made to the fact that three crucial steps – cholesterol side-chain cleavage, 17-hydroxylation, and

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aromatization – of the classical vertebrate steroid biosynthetic pathway are either absent or have very weak activity in molluscs (Fodor et al., 2020a). Most importantly, the homologues of the enzymes that catalyse the first and third of these reactions in vertebrates, as well as the functional sex steroid receptors, have so far not been found in molluscan genomes (Fodor et al., 2020a). Yet some papers have been published claiming that molluscs do seem affected (i.e., their physiology is potentially affected) by sex steroids occurring in the surface waters, though the robustness of the bioassay data in most of these papers has been questioned (reviewed by Scott, 2013). Clearly, even if there is an effect, it must take place via non-specific interactions (e.g., with receptors for other compounds) (Fodor et al., 2020a; Scott, 2012). This gives an additional reason to want to identify the targets of these molecules. Reference must be made to a recent suggestion that there is an alternative/"backdoor" pathway for vertebrate sex steroid synthesis (Miller & Auchus, 2019). This, however, is only about a suggested alternative pathway specifically for 5α-dihydrotestosterone synthesis in vertebrates and, it must be emphasized very strongly, not for testosterone or estrogen synthesis.

To further increase our knowledge on the endocrine system in molluscs as well as to provide further evidence for the notion that their endocrinology differs from the well-characterized vertebrate endocrine system, we have sequenced the whole transcriptome of the central nervous system (CNS) of the widely recognised molluscan model species, the great pond snail (*Lymnaea stagnalis*). Subsequently, we screened the transcriptome and found homologues of some of the genes that are related to those involved in the vertebrate sex steroidogenesis pathway, but did not find some of the key transcripts nor any sex steroid receptor-relevant sequences.

MATERIALS AND METHODS

Maintenance of *L. stagnalis* culture, nucleotide sequencing, and bioinformatics were conducted as previously described (Fodor et al., 2020c, 2020d). Briefly, for nucleotide sequencing, 5 months old, mature specimens of *L. stagnalis* were used from our laboratory-bred stocks. Snails were kept in large holding tanks containing 10 L oxygenated artificial snail water with low copper content at a constant temperature of 20°C (± 1.5°C) on light:dark regime of 12 h:12 h. Specimens were fed on lettuce ad libitum three times a week. All procedures on snails were performed according to the protocols approved by the Scientific Committee of Animal Experimentation of the Balaton Limnological Institute (VE-I-001/01890-10/2013).

The whole CNS was dissected from the snails (n = 10) and homogenized using a TissueLyser LT (QIAGEN) in TRI reagent (#93289, Sigma-Aldrich). RNA was isolated with Direct-zol™ RNA MiniPrep (#R2050, Zymo Research) following manufacturer's instructions. The RNA was quantified by Qubit BR RNA Kit (#Q10211, ThermoFisher) and the quality was checked on Agilent Bioanalyzer 2100 using RNA 6000 Nano Kit (#5067-1511, Agilent).

Nanopore sequencing was used for identification of homologous sequences to vertebrate sex steroidogenesis pathway and sex steroid receptor-relevant genes in the whole transcript. The library was prepared using cDNA-PCR Kit (#SQK-PCS108, Oxford Nanopore Technologies) according to the description from the manufacturer. The sample was sequenced on a MinION device with R9.4.1 flowcells (#FLO-MIN106). Base calling was performed using Guppy v3.2.2 software. Previous findings on relevant sequences of *Aplysia californica*, *Biomphalaria glabrata*, *Crassostrea gigas* and *Mizuhopecten yessoensis* (Adema et al., 2017, Thitiphuree et al., 2018), as well as relevant vertebrate sequences, were used as search queries (details are presented in Appendix 1). Adapters were trimmed with Porechop v0.2.4 (Wick, 2018), moreover sequences with internal adapters, which were indicating chimera reads, were also split with Porechop. Reads were assembled with CLC Genomics Workbench v12.0.3 software *de novo* pipeline (QIAGEN). Consensus sequence was called and manually corrected within CLC Genomics Workbench. For verification and sequence correction, the findings were compared with virtual cDNA sequences extracted from the unannotated genomic data (generated by Illumina sequencing) to which we have access as part of the *L. stagnalis* genome consortium (genome publication in preparation). The identified sequences were submitted to the NCBI Nucleotide database. Conserved domain search using NCBI CDD/SPARCLE was performed to check if the key regions were present in the deduced protein sequences.

TABLE 1. Identified *L. stagnalis* homologs with NCBI accession numbers to genes involved in the vertebrate sex steroidogenesis pathway and receptor-mediation of vertebrate sex steroids.

Vertebrate sex steroidogenesis pathway enzymes and sex steroid receptors	Abbreviation / Synonyms	Homolog(s) in *L. stagnalis*	Reference	NCBI accession #
Steroidogenic acute regulatory protein	StAR / STARD1	found	this study	MT655302
Side-chain cleavage enzyme	CYP11A	not found	-	-
3α-hydroxysteroid dehydrogenase	3α-HSD	not found	-	-
3β-hydroxysteroid dehydrogenase	3β-HSD	found	this study	MT655303
17α-hydroxylase/17,20-lyase	CYP17	found	this study	MT655304
17β-hydroxysteroid dehydrogenase	17β -HSD	found	this study	MT643176; MT655305; MT655306; MT655307
5α-reductase	-	found	this study	MT643177; MT643178
Aromatase	CYP19A	not found	-	-
Nuclear estrogen receptor alpha	nERα / NR3A1	found	Fodor et al., 2020a	MN989918
Nuclear progesterone receptor	nPR / NR3C3	not found	-	-
Nuclear androgen receptor	nAR / NR3C4	not found	-	-
Progesterone receptor membrane component 1	PGRMC1	found	Fodor et al., 2020a	MT178274
Steroid binding globulins	SBGs	not found	-	-

RESULTS AND DISCUSSION

Among the molluscan species, *L. stagnalis* has been investigated in most detail in terms of neuroendocrinology and reproduction (Benjamin & Kemenes, 2013; Di Cristo & Koene, 2017; Fodor et al., 2020b; Koene et al., 2010; Ladadic et al., 2007; Pirger et al., 2018), but this is the first study to comprehensively examine whether sex steroidogenesis-related sequences are present in this species.

The results of our screening are presented in Table 1. Regarding enzymes involved in the vertebrate sex steroidogenesis pathway, we did not find any homologous sequence to vertebrate genes coding for CYP11A (side-chain cleavage enzyme), CYP19A (aromatase), and 3α-HSD (3α-hydroxysteroid dehydrogenase). However, we did find homologs to StAR (steroidogenic acute regulatory protein), 3β-HSD (3β-hydroxysteroid dehydrogenase), CYP17 (17α-hydroxylase/17,20-lyase), 17β–HSD (17β-hydroxysteroid dehydrogenase), and 5α-reductase. In contrast, we could not identify any homologs to sex steroid receptor genes nPR (nuclear progesterone receptor) and nAR (nuclear androgen receptor) of vertebrates. Moreover, we did not find any homologs to SBGs (steroid-binding globulins). The characteristic motifs of relevant vertebrate homologous sequences could be identified in all of the identified homolog sequences. For example, the SRPBBC domain and the P450 domain for StAR and CYP17, respectively (Appendix 2).

As shown in Figure 1, although *L. stagnalis* has some of the homologs that are involved in the steroid biosynthetic pathway in vertebrates, the transcript of two key genes (CYP11A and CYP19A) that are pivotal to accomplish the conventional vertebrate-type sex steroid synthesis are not present in transcriptome data of this species.

Regarding oxidoreductase reactions, we could identify homologous sequences to 3β-HSD, 17β-HSD, and 5α-reductase. These sequences are also present in other molluscan species including, for example, *Biomphalaria glabrata*, *Aplysia californica*, and *Mizuhopecten yessoensis* (Adema et al., 2017; Thitiphuree et

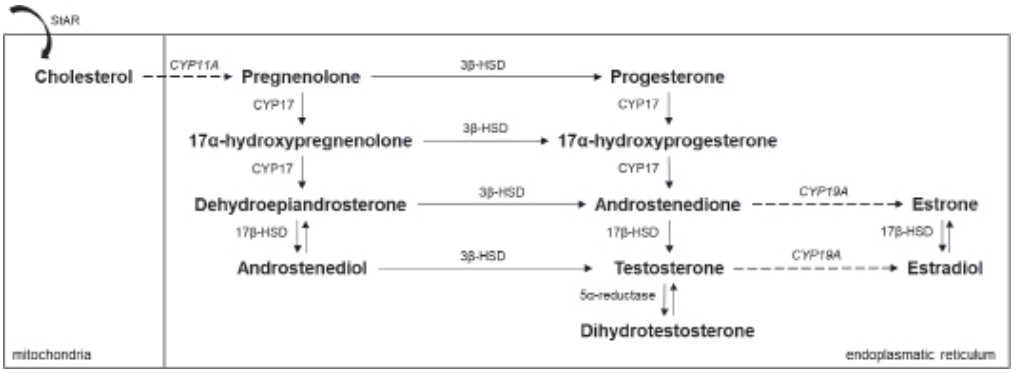

FIG. 1. The putative pathway for the conventional sex steroid synthesis in *L. stagnalis*. Six reactions/processes (solid arrows), though some with low activity, and the enzymes that catalyse them appear to be present in *L. stagnalis*. We neither found evidence for the presence of the transcripts for CYP11A and CYP19A (marked in italics), this supports the notion that two crucial steps – cholesterol side-chain cleavage and aromatization (dashed arrows) – of the classical vertebrate steroid biosynthetic pathway are absent in molluscs.

al., 2019). Furthermore, the reactions carried out by these enzymes seem to be also present in *L. stagnalis* – for example, a radioactive tracer study demonstrated that it can perform the conversion of pregnenolone to progesterone (de Jong-Brink et al., 1981). However, it should be pointed out that functional studies are required to validate that these reactions form part of a steroid biosynthetic pathway which is equivalent to that of vertebrates. This is because the presence of these homologs in molluscs does not mean that they have a similar function, i.e. steroids may not necessarily the intended substrate of these enzymes in invertebrates. For example, a recent paper investigating two freshwater gastropods implicated the existence of alternative 5α-reductase substrates most likely involved in the development of shell morphology (Baynes et al., 2019).

Concerning CYP enzymes, in agreement with other molluscs, our transcript analysis supports that CYP17 is present in *L. stagnalis* and can potentially perform, although seemingly at a very low level, the conversion of progesterone to androstenedione (reviewed by Fernandes et al., 2010; Scott, 2012). However, the lack of homolog transcripts to CYP11A and CYP19A is not so surprising. Only a "partial protein equivalent" to CYP11A has been tentatively identified in molluscs so far (Blalock et al., 2018). The precursor cholesterol is undoubtedly present in *L. stagnalis* (Altelaar et al., 2005) and we could identify a homolog to the StAR protein, that is responsible for the transport of cholesterol into the inner membrane of mitochondria and therefore essential to the formation of pregnenolone (Stocco, 2000). This indicates that cholesterol has the potential to be utilized as a resource for steroid synthesis. At the same time, there is no study to demonstrate proven side-chain cleavage activity either in this species or in molluscs in general. However, it should be pointed out that the unanswered question is whether pregnenolone can be absorbed from the water and used as a progesterone precursor by molluscs, but to the authors' best knowledge there is no study demonstrating this.

Similar to CYP11A, the gene for the enzyme CYP19A has not yet been identified outside of vertebrates (reviewed by Fodor et al., 2020a). Clearly, these two genes seem specific to vertebrates and their immediate ancestors: the current theory is that the evolution of side-chain cleavage in basal vertebrates was possibly the driver for the co-evolution of a dedicated aromatizing enzyme (i.e., CYP19A) in vertebrates (Markov et al., 2017). Although an *in vivo* study has recently demonstrated estrogen production by a bivalve mollusc (Hallmann et al., 2019), the yields were very low (< 0.1%), suggesting it was more likely to be due to the now well-recognized phenomenon of enzyme promiscuity (Atkins, 2015; Lathe et al., 2015) rather than the presence of a specific 'alternative' aromatase-like enzyme. In order to try

and fill in the gaps in the pathway in molluscs, the suggestion has been made that two P450 enzymes that are known to be present in molluscs, known as CYP10 and CYP3A, fill in the roles of CYP11A and CYP19A, respectively (Thitiphuree et al., 2019). However, it must be emphasized that, at the moment, this is pure speculation. Even if these enzymes did actually have the suggested activities (probably as a result of "enzyme promiscuity" mentioned above), the yields are likely to be far too low to maintain a sex steroid-based hormonal system. To note, CYP10 was previously identified in *L. stagnalis* (Teunissen et al., 1992) and our transcriptomic data also supports its presence.

In terms of sex steroid receptors, our results showed that homolog transcripts to genes for nAR and nPR are not present in *L. stagnalis*. This is not surprising, as the current theory is that these genes did not evolve until late in vertebrate evolution (Markov et al., 2017; Thornton, 2001). That is to say, similar to CYP11A and CYP19A, the assumption that nAR and nPR are present in molluscs is highly unlikely from a genomic (and transcriptomic) viewpoint. In our previous study, the coding sequence of a homolog to the human nuclear estrogen receptor alpha was determined in *L. stagnalis* (Fodor et al., 2020a), but so-called "molluscan estrogen receptors" have been shown not to bind estrogen, that is, they are not functional estrogen receptors (Bannister et al., 2013; Kajiwara et al., 2006; Keay et al., 2006; Ma et al., 2019; Markov et al., 2008, 2009; Matsumoto et al., 2007; Pirger et al., 2018; Thornton et al., 2003). In the same study, a homologous sequence to PGRMC1 was also determined in *L. stagnalis*. This molecule has been shown to act as a chaperone protein for a membrane progesterone receptor in vertebrates but does not actually bind progesterone itself (Cahill et al., 2016; Gonzalez et al., 2020; Pang et al., 2013; Ren et al., 2019; Thomas et al., 2007, 2014). In most vertebrates, SBGs (steroid binding globulins) are found and shown to bind testosterone and estradiol with high affinity and specificity. Their binding properties are very similar to those of nuclear receptors and they could theoretically be potential steroid receptor candidates in molluscs. However, we did not find any homologs to these molecules in *L. stagnalis*.

In summary, transcriptomic data from *L. stagnalis* revealed the absence of several key sequences and/or the lack of gene expression of several key genes that are essential to accomplish the classical vertebrate-type sex steroid synthesis, as well as the receptor-mediation of sex steroids. It should be noted that the analysis was conducted on the CNS and did not cover the periphery (e.g., ovotestis, reproductive glands) so it remains possible that 3α-HSD and SBGs are only expressed peripherally, and hence not detected in this study. Therefore, future research should aim to include the reproductive organs. Nevertheless, one must question whether natural selection in molluscs would have likely favoured the evolution of a steroid-based endocrine system. Our findings support the notion that molluscan endocrinology differs from the well-characterized vertebrate endocrine system.

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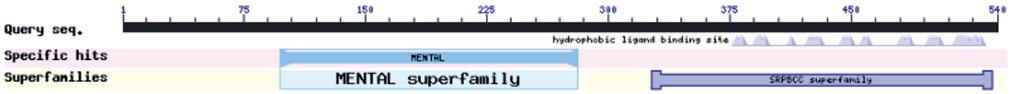
APPENDIX 1. Invertebrate and vertebrate sequences with NCBI accession numbers that were used to query in our transcriptome analysis.

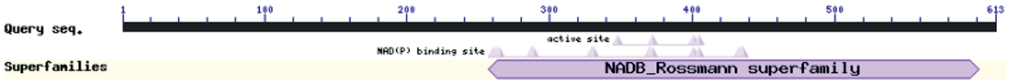
Query sequence	Species	Accession number
StAR-related lipid transfer protein 3 (StAR3)	*Mizuhopecten yessoensis*	AXY92157.1
StAR-related lipid transfer protein 3 (StAR3)	*Crassostrea gigas*	XP_011434195.2
StAR-related lipid transfer protein 3 (StAR3)	*Rattus norvegicus*	NP_001014251.1
3α-hydroxysteroid dehydrogenase (3α-HSD)	*Rattus norvegicus*	P23457.1
3β-hydroxysteroid dehydrogenase (3β-HSD)	*Mizuhopecten yessoensis*	AXY92162.1
3β-hydroxysteroid dehydrogenase (3β-HSD)	*Crassostrea gigas*	XP_011433629.1
3β-hydroxysteroid dehydrogenase (3β-HSD)	*Rattus norvegicus*	NP_001007720.3
17α-hydroxylase/17,20-lyase (CYP17)	*Aplysia californica*	XP_012941324.1
17α-hydroxylase/17,20-lyase (CYP17)	*Mizuhopecten yessoensis*	AXY92158.1
17α-hydroxylase/17,20-lyase (CYP17)	*Crassostrea gigas*	NP_001292231.1
17α-hydroxylase/17,20-lyase (CYP17)	*Rattus norvegicus*	NP_036885.1
17β-hydroxysteroid dehydrogenase 8 (17β-HSD8)	*Aplysia californica*	XP_005103566.1
17β-hydroxysteroid dehydrogenase 8 (17β-HSD8)	*Mizuhopecten yessoensis*	MH040338.1
17β-hydroxysteroid dehydrogenase 8 (17β-HSD8)	*Crassostrea gigas*	XP_011430925.1
17β-hydroxysteroid dehydrogenase 8 (17β-HSD8)	*Rattus norvegicus*	NP_997694.1
17β-hydroxysteroid dehydrogenase 11 (17β-HSD11)	*Mizuhopecten yessoensis*	MH040339.1
17β-hydroxysteroid dehydrogenase 11 (17β-HSD11)	*Rattus norvegicus*	NP_001004209.1
17β-hydroxysteroid dehydrogenase 14 (17β-HSD14)	*Aplysia californica*	XP_012939218.1
17β-hydroxysteroid dehydrogenase 14 (17β-HSD14)	*Mizuhopecten yessoensis*	AXY92161.1
17β-hydroxysteroid dehydrogenase 14 (17β-HSD14)	*Crassostrea gigas*	XP_011423932.1
17β-hydroxysteroid dehydrogenase 14 (17β-HSD14)	*Rattus norvegicus*	NP_001178040.1
5α-reductase 1	*Crassostrea gigas*	EKC36980.1
5α-reductase 2	*Aplysia californica*	XP_005112214.1
Aromatase (CYP19A)	*Mus musculus*	NP_001335102.1
Aromatase (CYP19A)	*Gallus gallus*	NP_001351628.1
Aromatase (CYP19A)	*Danio rerio*	NP_571229.3
Nuclear androgen receptor (nAR)	*Homo sapiens*	P10275.3
Nuclear androgen receptor (nAR)	*Mus musculus*	P19091.1
Nuclear androgen receptor (nAR)	*Gallus gallus*	NP_001035179.1
Nuclear androgen receptor (nAR)	*Danio rerio*	NP_001076592.1
Nuclear progesterone receptor (nPR)	*Homo sapiens*	P06401.4
Nuclear progesterone receptor (nPR)	*Mus musculus*	Q00175.2
Nuclear progesterone receptor (nPR)	*Gallus gallus*	NP_990593.1
Nuclear progesterone receptor (nPR)	*Danio rerio*	NP_001159807.1
Corticosteroid-binding globulin (CBG)	*Mus musculus*	NP_031644.1
Sex hormone-binding globulin (SHBG)/ sex steroid-binding globulin (SSBG)	*Mus musculus*	P97497.1

APPENDIX 2. Identified conserved domains (specific hits) within the *L. stagnalis* homolog protein sequences (query sequence) for in silico validation. Sequence accession numbers are indicated in Table 1. Conserved domain search was performed with NCBI CDD/SPARCLE (Lu et al., 2020; Marchler-Bauer et al., 2017).

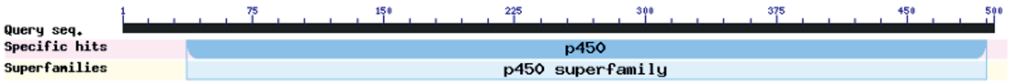
> Lymnaea stagnalis_StAR-related lipid transfer protein 3



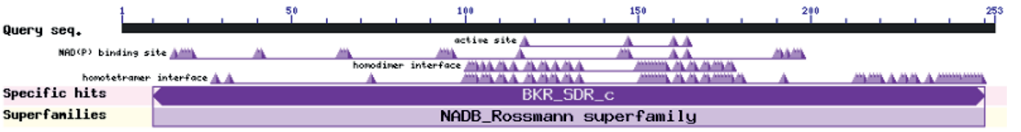
> Lymnaea stagnalis_3β-hydroxysteroid dehydrogenase



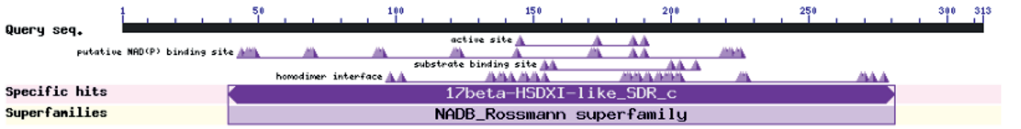
> Lymnaea stagnalis_17α-hydroxylase/17,20-lyase



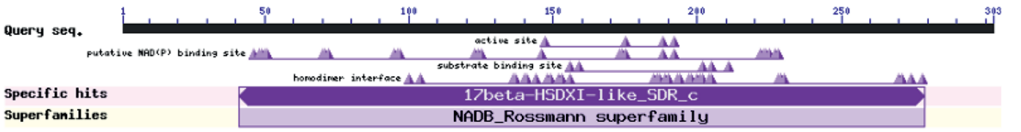
> Lymnaea stagnalis_17β-hydroxysteroid dehydrogenase 8



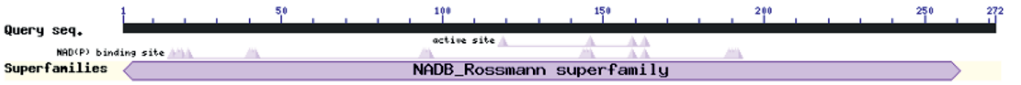
> Lymnaea stagnalis_17β-hydroxysteroid dehydrogenase 9



> Lymnaea stagnalis_17β-hydroxysteroid dehydrogenase 11



> Lymnaea stagnalis_17β-hydroxysteroid dehydrogenase 14



> Lymnaea stagnalis_5α-reductase 1

> Lymnaea stagnalis_5α-reductase 2