

# Proteins of penial mamilliform glands in closely related *Littorina* species (Mollusca, Caenogastropoda): variability and possible contribution to reproductive isolation

Arseniy Lobov<sup>1,2</sup>, Irina Babkina<sup>1</sup>, Arina Maltseva<sup>1</sup>, Natalia Mikhailova<sup>3</sup>, and Andrey Granovitch<sup>1</sup>

<sup>1</sup>Department of Invertebrate Zoology, Faculty of Biology, Saint Petersburg State University, Universitetskaya nab., 7–9, Saint Petersburg, 199034, Russian Federation

<sup>2</sup>Laboratory of Regenerative Biomedicine, Institute of Cytology, Russian Academy of Sciences, Tikhoretskiy pr., 4, Saint Petersburg, 194064, Russian Federation

<sup>3</sup>Cell Technologies Center, Institute of Cytology, Russian Academy of Sciences, Tikhoretskiy pr., 4, Saint Petersburg, 194064, Russian Federation

Address correspondence and requests for materials to Arseniy Lobov, arseniylobov@gmail.com

## Abstract

The forces driving reproductive isolation emergence during the process of sympatric speciation are still intensely debated. Mechanisms of gametic isolation (which are known to form rapidly in several models) take the central place in these debates. Nevertheless, the approximative capacity of a few investigated models to other taxa could be questioned, generating demand for the adoption of additional model organisms to study sympatric speciation. The group of closely related species of the genus *Littorina* (subgenus *Neritrema*) sympatrically inhabiting seashores are promising. In this study, we performed comparative proteomic analysis of penial tissues of four *Neritrema* species to identify potential effectors contributing to gametic isolation. Among 272 analyzed proteins, 13 mamilliform gland-specific proteins (possibly transferred to the female during copulation) were detected, as well as five proteins specifically expressed in the epithelium of the penial basal part. Eight of these proteins were species-specific and may be involved in the maintenance of reproductive barriers.

**Keywords:** *Littorina*, proteomics, gamete recognition proteins, mamilliform gland, reproductive isolation, gametic isolation, Mollusca, 2D-DIGE.

## Introduction

Reproductive isolation is regarded as a key species criterion within the biological species concept (De Queiroz, 2007). When reproductive barriers emerge without geographic separation, subpopulations appear under the sympatric (as opposed to allopatric) speciation model (Bolnick and Fitzpatrick, 2007; Butlin et al., 2008). Although the mechanisms of sympatric speciation have been actively investigated in the last 30 years, they remain vague in many aspects. One of the possible mechanisms is post-copulatory prezygotic reproductive (gametic) isolation due to incompatibility of molecules involved in fertilization (Swanson and Vacquier, 2002; Matute, 2010; Lobov et al., 2019).

There are few model systems to study gametic isolation, mainly in species of animals with external fertilization (Wilburn and Swanson, 2016). Model organisms with internal fertilization have until recently included only insects and mammals (Vieira and Miller, 2006; Wilburn and Swanson, 2016). A group of cryptic species of genus *Littorina* (Mollusca, Caenogastropoda) is a newly elaborated model system to analyze gametes incompatibility among internal fertilizers. The subgenus *Neritrema* comprises two groups of closely related species: “saxatilis” (*L. saxatilis* (Olivi 1792),

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**Authors' information:** Arseniy Lobov, PhD student, Junior Researcher, [orcid.org/0000-0002-0930-1171](https://orcid.org/0000-0002-0930-1171); Irina Babkina, student, [orcid.org/0000-0002-0406-7021](https://orcid.org/0000-0002-0406-7021); Arina Maltseva, PhD, Associate Professor, [orcid.org/0000-0003-1973-4728](https://orcid.org/0000-0003-1973-4728); Natalia Mikhailova, Dr. of Sci. in Biology, Leading Researcher, [orcid.org/0000-0003-1650-9330](https://orcid.org/0000-0003-1650-9330); Andrey Granovitch, Dr. of Sci. in Biology, Professor, [orcid.org/0000-0002-5203-104X](https://orcid.org/0000-0002-5203-104X)

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*L. arcana* Hannaford Ellis 1978, *L. compressa* Jeffreys 1865) and “obtusata” (*L. obtusata* (Linnaeus 1758), *L. fabalis* (Turton 1825)); all of these sympatrically inhabit the European North Atlantic sea shores (Yashenko and Granovitch, 2002; Granovitch et al., 2004). Physiological consequences of local ecological and evolutionary adaptation can be traced in particular species and subpopulations of these molluscs (Sokolova et al., 2000, 2001; Panova and Johannesson, 2004; Rolán-Alvarez et al., 2015; Maltseva et al., 2016). This suggests the importance of ecological sympatric speciation in the evolutionary history of this species group (Johannesson, 2003; Grahame et al., 2006; Rolán-Alvarez et al., 2007, 2015).

All representatives of *Neritrema* demonstrate a species-specific pattern of mating, but there is evidence that *L. saxatilis*/*L. arcana* and *L. obtusata*/*L. fabalis* may hybridize in nature (Mikhailova et al., 2009; Granovitch et al., 2013; Costa et al., 2020), and that interspecies copulations occur commonly in *L. saxatilis*/*L. arcana* (Mikhailova et al., 2008; unpublished data). Such a case of incomplete reproductive isolation along with certain features of *Neritrema* periwinkles (e.g., polyandry, sympatry, reinforcement and sexual conflict) serve as preconditions for gametic isolation between the species (Zigler et al., 2005; Kvarnemo and Simmons, 2013; Lobov et al., 2019). Thus, postcopulatory prezygotic reproductive barriers may be expected to contribute to the maintenance of species identities in this species group in sympatry.

In 2013 we launched the project devoted to the analysis of gamete interaction proteins involved in gametic isolation between *Littorina* species. We assumed that such proteins may have three possible origins in a male (Supplement, SM 1): testis and seminal vesicles (mature eu- and paraspermatozoa), prostate gland (seminal fluid) and penial mamilliform glands. We have characterized the species-specific paraspermal protein LOSP (Lobov et al., 2015, 2018); the analysis of prostate proteomes is in progress; and here we describe the results of interspecies proteomic comparison of mamilliform penial glands secretions.

The precise function of penial glands is unknown; it was suggested that they anchor the penis within the bursa copulatrix during copulation (Buckland-Nicks and Worthen, 1992). Each penial gland consists of four types of secretory cells: cells of multicellular gland (MGC), apocrine secretion cells and two types of mucous cells (Buckland-Nicks and Worthen, 1992; Ganzha et al., 2006). MGCs have extensive rough endoplasmic reticulum, so most likely these cells produce protein secretions. Hypothetically, these secretions may contribute to sperm conditioning and affect sperm viability, and thus could be involved in reproductive isolation. To test this assumption we aim to identify some species-specific mamilliform gland proteins. Using a gel-based

proteomic approach followed by mass-spectrometric analysis, we compared proteomes of mamilliform glands and basal fragments of penis from *L. saxatilis*, *L. arcana*, *L. obtusata* and *L. fabalis*.

## Material and Methods

**Samples** Males of *L. saxatilis*, *L. arcana*, *L. obtusata* and *L. fabalis* were collected from the sympatric Norwegian populations (70°17'00.0"N 30°58'40.9"E). The procedure of species identification was described before (Granovitch et al., 2008). Penises were dissected and divided into two parts: mamilliform glands and basal fragments (SM 1). Each piece of tissue was collected into lysis buffer and homogenized (Maltseva et al., 2016).

**2D DIGE** Samples were conjugated with Cy2, Cy3 or Cy5 fluorophores (Luminoprobe). Three samples were separated in one gel. There were two biological replicates with at least two technical replicates including Cy-fluorophore-swap for each sample. Isoelectric focusing was carried out with IPG ReadyStrip (18 cm or 7 cm, pH 3–10, Bio-Rad) in a Protean IEF Cell (Bio-Rad) following the manufacturer's recommendations. The second direction of electrophoresis was performed in 15% PAAG with a tris/glycine/SDS buffer system (BioRad; Laemmli, 1970). Electrophoregrams were visualized using Typhoon FLA 9500 (GE Healthcare).

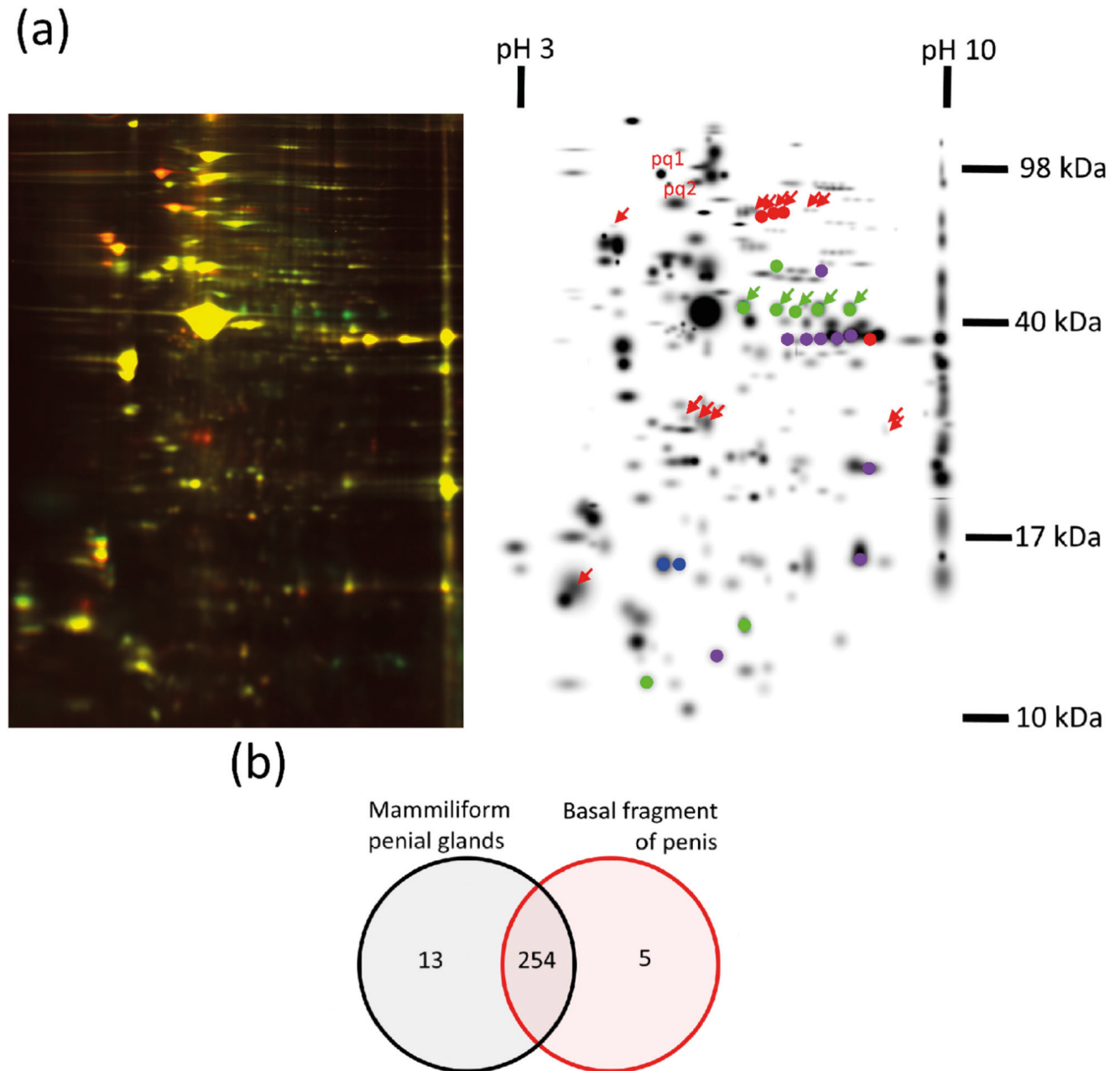
**Statistical analysis** Spot identification for 2D electrophoresis analysis was done in PDQuest (BioRad) software. The final dataset was analyzed in R (R Core Team, 2019; Maltseva et al., 2016).

**LC-MS/MS** Differentially expressed proteins were excised from the Coomassie stained gels and identified by HPLC-tandem-mass-spectrometry (Agilent 6538, Agilent Technologies) following the “bottom up” approach as described earlier (Maltseva et al., 2016). MS/MS search was done using Agilent Spectrum Mill MS Proteomics Workbench (Agilent Technologies; Rev B.04.00.127) against the *L. obtusata* (Marques et al., 2019), *L. fabalis* (Marques et al., 2019), *L. saxatilis* (Marie Westram et al., 2018), *L. littorea* (Gorbushin, 2018) transcriptomes and the TrEMBL database for Mollusca [6447] database (2019; 396079 proteins; <https://www.uniprot.org/uniprot/?query=taxonomy:6447%20reviewed:no>).

All the technical details of the analysis are present in Supplement 2 (SM 2).

## Results and Discussion

Among 272 protein spots analyzed, the majority were shared among all species and tissues, but there were several species- and tissue-specific proteins (Fig. 1, a–b; SM 3).



**Fig. 1.** Penial proteomes of *Littorina* species. (a) Left — example of 2D-DIGE electrophoregram of mamilliform gland (red) and basal part of penis (green) of *L. saxatilis* in two merged channels; Right — master gel based on all analyzed 2D-electrophoregrams; Arrows — tissues-specific proteins: red — mamilliform gland specific proteins, green — proteins from the basal part of penis named f\_1–f\_5; Dots — species-specific proteins: green — *L. saxatilis/L. arcana*, red — *L. obtusata*, blue — *L. fabalis*, purple — *L. obtusata/L. fabalis*; pq1 and pq2 — proteins with higher abundance in mamilliform gland. (b) — Venn diagram with the numbers of tissue-specific proteins.

### Tissue-specific proteins

There were 13 mamilliform gland-specific and five basal penial fragment-specific proteins (Fig. 1). Although our MS-data were of fine quality in terms of spectra number and intensity, we did not succeed in identifying these tissue-specific proteins. We also found several proteins much more abundant in mamilliform gland samples (pq1 and pq2) and identified only one of them (Fig. 1): pq1 as endoplasmin (Hsp90b1) based on the TrEMBL

database. Identification of pq1 protein had maximal score when searched against the TrEMBL database. If available databases for the *Littorina* species were used, pq1 was still identified as Hsp90b1 (SM 3).

Hsp90b1 is a chaperone functioning as a folding protein in the endoplasmic reticulum (ER) (Chen et al., 2005). Hsp90b1 has been proven to be a rather conservative component of cellular secretory machinery. This fact corresponds well with its predominant presence in mamilliform glands samples (Buckland-Nicks and



Worthen, 1992). In agreement with its evolutionary conservativeness, no interspecies variability was revealed.

The most probable reason for our failure to identify other proteins is the absence of reproductive proteins in the available databases (Lobov et al., 2015).

### Species-specific proteins

Three mamilliform gland-specific proteins were species-specific for *L. obtusata* (Fig. 1; SM 4). They have similar MW and pI, and probably represent different isoforms of the same protein. Their function is unknown, and does not necessarily have to be “house-keeping” (like p<sub>q1</sub> and p<sub>q2</sub>). It is still plausible that at least some of the mamilliform gland-specific proteins are secreted during copulation and perform some reproduction-specific functions.

Among other species-specific proteins detected here, many were described earlier as proteins expressed in tissues other than penis. However, basal fragment-specific proteins f<sub>1</sub>–f<sub>5</sub> (Fig. 1, a — green arrows with dots; SM 4) were previously detected as both species- and penis-specific (in comparison to other body parts); mamilliform glands were proposed as the site of their production (Maltseva et al., 2016). Here we confirmed the specificity of f<sub>1</sub>–f<sub>5</sub> for the *L. saxatilis* and *L. arcana* species pair, but disproved their origin from mamilliform glands; instead, they are expressed in the basal part of the penis. Most probably, their production is related to small glands (each composed of two to three cells) in the epithelium of the penial basal part plus filament and/or penial sperm groove (Ganzha et al., 2006). If this is the case, they might be involved in sperm conditioning after insemination or penis anchoring during copulation. The first guess implies their possible involvement in gamete recognition and, subsequently, in reproductive isolation between the “*L. saxatilis* + *L. arcana*” pair and other species.

### Conclusion

We conducted proteomic analysis of mamilliform penial glands and basal fragments of the penis in four species of genus *Littorina*. Among 272 analyzed proteins, 13 penial gland-specific proteins may be involved in the secretion process or may be secreted by reproduction-related tissues; 5 proteins are specifically expressed in the penial basal part. Three mamilliform gland-specific proteins were species-specific for *L. obtusata*, while all five basal part-specific proteins (probably produced on penial and/or sperm groove epithelium) were specific for *L. saxatilis*/*L. arcana*. Based on their localization and species-specificity, these eight proteins may contribute to reproductive barriers, but we have no confirmation yet. In this context, analysis of prostate proteins (components of seminal fluid), which is currently in progress,

seems most prospective for disentangling mechanisms of postcopulative reproductive barriers in these periwinkles.

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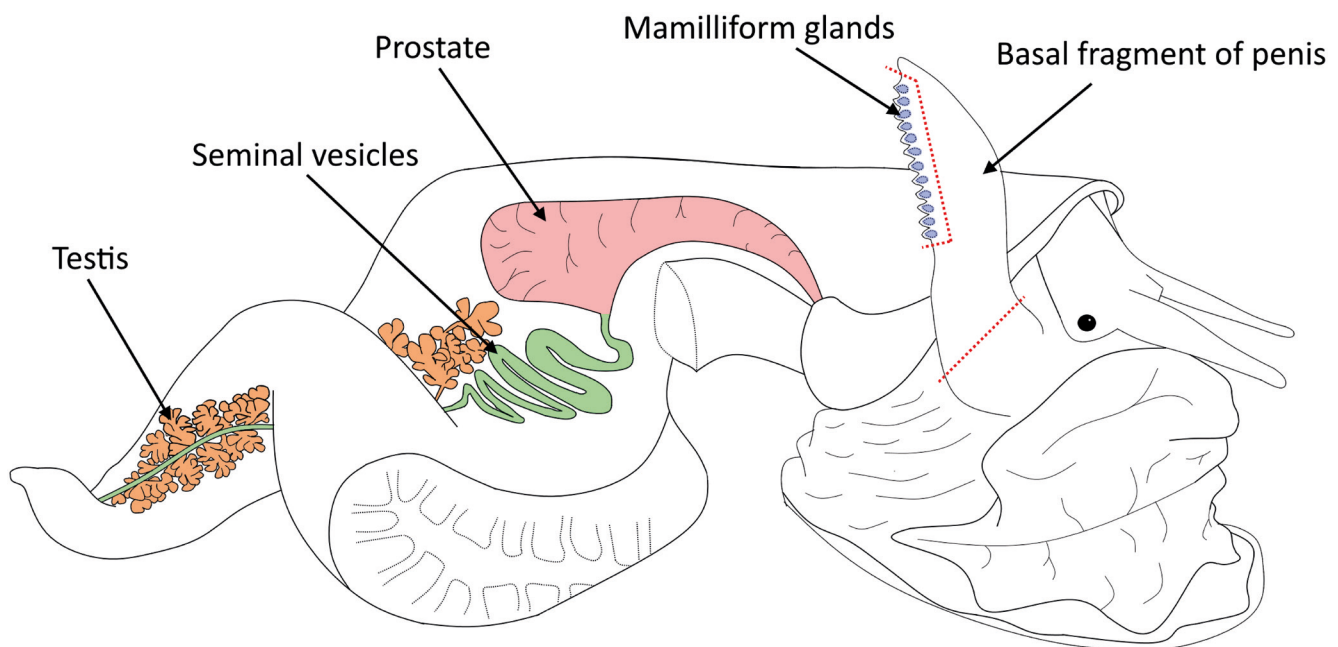
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## SUPPLEMENTS

**SM 1 — Scheme of *Littorina* male's anatomy;  
indicates tissues assumed to produce gamete recognition proteins**

Testis (yellow) — the tissue where eu- and parasperms are formed; seminal vesicle (green) — the channel where eu- and parasperms are stored before insemination; prostate (pink) — the tissue where seminal fluid proteins undergo synthesis, maturation and exocytosis; mamilliform glands (blue) — specialized glands of unknown function on the penis; basal fragment of penis — the part used as a sample of comparison for identification of mamilliform gland-specific proteins; red dotted line — the border of penis dissection during sample preparation.



## SM 2 — Electrophoresis and protein identification procedures

In total, 16 different samples of four species (*L. saxatilis*, *L. arcana*, *L. fabalis*, *L. obtusata*) representing two different parts of a penis (mamilliform glands or basal fragment of a penis) in two biological replicates were subjected to gel-based proteomic analysis. Every sample was a “pooled one”, means contained biological material of six individuals, to increase its biological representativeness.

The body parts were put to the lysis buffer (7M Urea, 2M Thiourea, 4% CHAPS, 30 mM Tris pH 8,0), frozen in liquid nitrogen, and then homogenized by RETSCH Mixer Mill MM 400 for 20 min at 30 Hz. Prior to 2D-DIGE electrophoresis every sample was analyzed in 1D electrophoresis for quality control and protein concentration estimation.

Each sample was analyzed in 2D-DIGE at least twice being conjugated with different fluorophores and in combination with different counter samples for comparison. Repeated runs of the same sample were considered as technical replicates ( $n = 2-4$ ). Prior to electrophoretic separation, proteins of each sample were conjugated with the fluorophores Cy2, Cy3 or Cy5 (Luminoprobe, BioDye) in a proportion of 400 pmol of Cy per 50  $\mu$ g of total protein. Samples mixed with Cy-fluorophores were incubated for 45 min on ice in the dark, the reaction was then stopped by the addition of 10  $\mu$ mol of L-lysine, and the samples were further incubated for 30 min under the same conditions.

The samples conjugated with the different Cy-fluorophores were pooled and loaded into an IPG ReadyStrip (18 cm for first biological replicate or 7 cm for second one, pH 3–10, BioRad) during passive rehydration (overnight at room temperature, in the dark). Separation in the first direction was carried out in the Protean IEF Cell (BioRad) using the method recommended by the manufacturer. Before separation in the second direction, IPG-strips with focused proteins were incubated in equilibration buffers (6 M urea, 2% SDS, 20% glycerin, 0.375 M tris, pH 8.8) for 15 min: first with 2% dithiothreitol and then with 2.5% iodoacetamide. Electrophoretic separation in the second direction was performed in the Protean xii cell (BioRad) for 18 cm IPG-strips or MiniProtean TetraCell (BioRad) for 7 cm IPG-strips in 15% PAAG in Tris/glycine/SDS buffer. The GE Typhoon 9500 FLA laser scanner (GE Healthcare) was used for visualization. Merging of the electrophoregrams was done using ImageJ 1.48 v (<http://imagej.nih.gov/ij>). For spots excision gels were stained by Coomassie Brilliant Blue G-250 (Amresco).

Protein identification was done in accordance to a ‘bottom up’ approach, which means tandem mass spectrometry (MS/MS) identification of tryptic peptides by database search. Spots of interest were excised from the

gel with a scalpel and cut into pieces. Gel pieces of each spot were destained with 50% acetonitrile in 25 mM Tris (pH 8.2), dehydrated in 100% acetonitrile and rehydrated in bovine trypsin solution (20 ng  $\mu$ l<sup>-1</sup>, 25 mM Tris, pH 8.2) on ice for 60 min. After rehydration, any excessive trypsin solution was removed, and gel pieces were covered with 25 mM Tris (pH 8.2) and incubated at 37°C overnight. Tryptic peptides were eluted with 50% ACN/ 0.1% FA, dried in vacuum concentrator CentriVap (Labconco) and dissolved in 10  $\mu$ l of 5% ACN/0.1 FA.

The samples were analyzed using liquid chromatography (LC; Agilent 1260) coupled with MS/MS (ESI-Q-ToF UHD 6538, Agilent Technologies). The gradient elution method was 10%B to 60%B for 40 min and further to 100%B (with corresponding decreasing of A) for 10 min, where B was 90% acetonitrile with 0.1% formic acid, A was 5% acetonitrile with 0.1% formic acid, and flow rate was 15  $\mu$ l min<sup>-1</sup>. We used a Zorbax SB-C18 column (5  $\mu$ m grain, 80 Å pores, 150  $\times$  0.5 mm; Agilent Technologies). An MS/MS search was carried out in the mode ‘Identity’ using Agilent Technologies Spectrum Mill MS Proteomics Workbench Rev B.04.00.127 software against free databases: *L. obtusata* (Marques et al., 2019), *L. fabalis* (Marques et al., 2019), *L. saxatilis* (Marie Westram et al., 2018), *L. littorea* (Gorbushin, 2018) transcriptomes and the TrEMBL database for Mollusca [6447] database (2019; 396079 proteins; [https://www.uniprot.org/uniprot/?query=taxonomy:6447%20 reviewed:no](https://www.uniprot.org/uniprot/?query=taxonomy:6447%20%20reviewed:no)); the precursor mass tolerance was set to  $\pm$ 20 ppm.

The validation procedure of identified proteins was performed with a minimum two unique peptides and a peptide false discovery rate (FDR) for validated proteins of 1%.

Qualitative and quantitative (was not presented in the article due to low informative value) gel analyses were carried out using PDQuest Advanced 8.0.1 software (BioRad). Normalization for the spot intensity estimates was done by total gel density. Spots with intensity values below the detection limit (i.e. not exceeding 0 relative to the background) were considered as absent for qualitative analysis. Spots were considered as reliably detected if they were detected in at least 2 technical replicates of the same sample, or in 1 technical replicate in at least 2 different samples. We classified potential post-translational modifications and/or splice variants as independent spot signals, because every particular modification and/or splice form possesses its own features and functions. In some cases, we know that these independent spot signals represent different forms of the same enzyme (like arginine-kinase or aldolase, see Maltseva et al., 2016), while in others, it might be suspected owing to similar molecular weight and subtle differences in isoelectric point.



### SM 3 — results of tryptic peptides MS/MS identification of pq1 and pq2

Despite rapid development of protein and peptide mass-spectrometry, *de novo* protein sequencing is still challenging. In routine laboratory practice, «bottom-up» analysis is the main approach for mass-spectrometry based protein identification (more information about «bottom-up» protein identification can be found elsewhere, e.g. in Zhang et al., 2013; in Maltseva et al., 2019 (in Russian), *etc.*).

The first step of this design is proteolysis (usually trypsinolysis). Depending on a sample, it can be the proteolysis of total protein extracts for LC-based proteomics or of just single protein spots after 2D-electrophoresis (as in this research). Then the tryptic peptides are analyzed using tandem mass-spectrometry with inclusion of peptide fragmentation process. Due to stochastic nature of fragmentation, the series of fragment are formed and analyzed to establish original peptide sequence. The peptide identification goes through comparison of obtained spectra with those predicted based on sequences from databases.

Following this strategy, we succeed in identification of only one penial-specific protein: pq1 (see the main text). Here are some details of the protein identification.

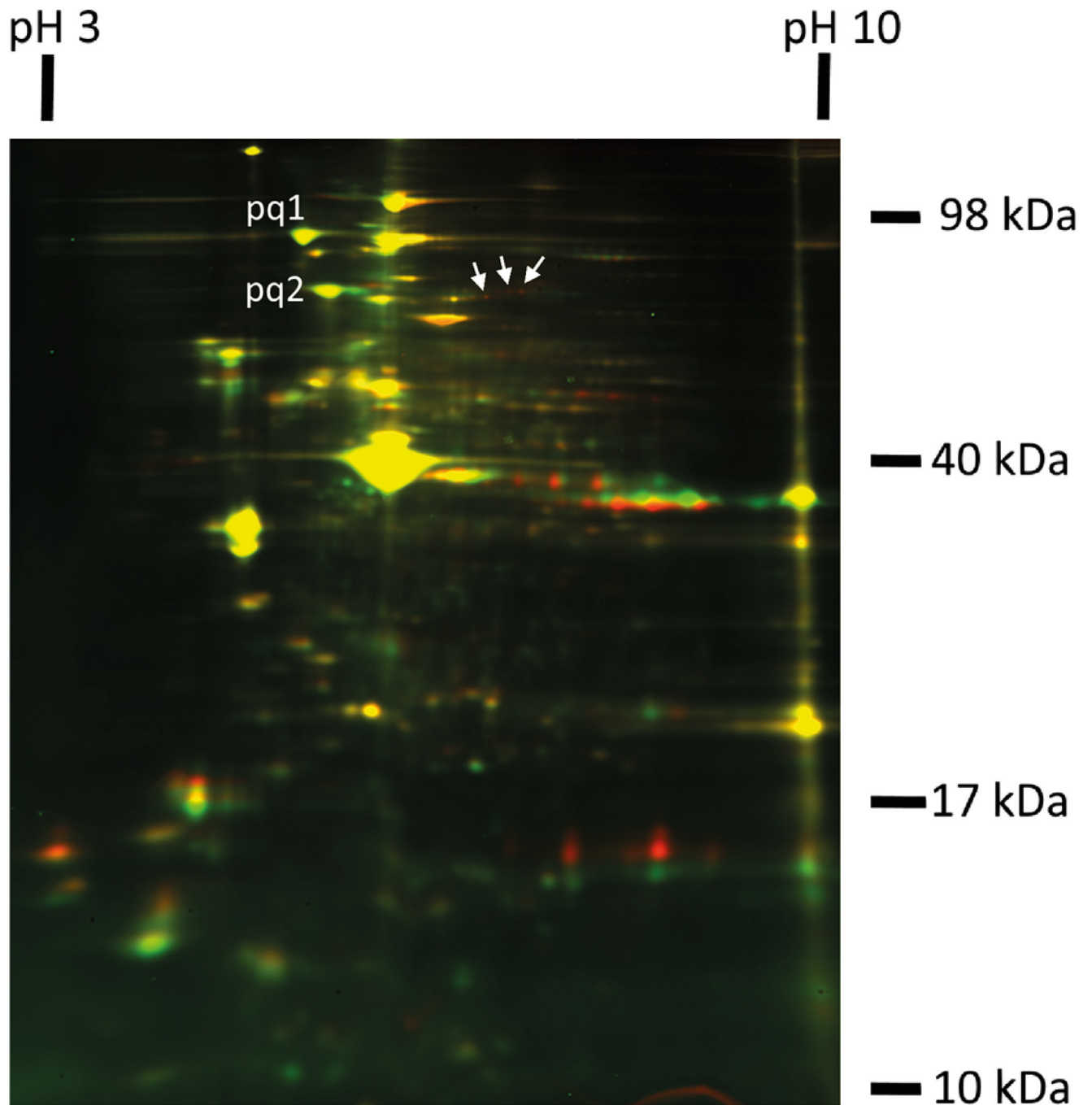
**SM\_Table 1. Results of pq1 MS/MS-identification using Agilent Spectrum Mill MS Proteomics Workbench using TrEMBL database for Mollusca [6447]. The only peptide identified by available databases for *Littorina* and corresponds to HSP90B1 orthologues in *L. fabalis* (TRINITY\_DN31682\_c1\_g1\_i1.p1; Marques et al., 2019), *L. obtusata* (TRINITY\_DN29853\_c1\_g3\_i1.p1; Marques et al., 2019), *L. saxatilis* (TR66798\_c0\_g1\_i1 | m.32098 & TR63539\_c3\_g9\_i1 | m.28446; Marie Westram et al., 2018), *L. littorea* (GenBank accession: GGCG01061570.1; Gorbushin, 2018) is shown in green**

Name	Accession number	Species	Protein MW (Da)	Score	Unique peptides	% AA coverage	Total spectral intensity	Number of spectra
Endoplasmin	A0A210PZR8	<i>Mizuhopecten yessoensis</i> (Yesso scallop)	91323.1	70.76	9	5.2	2.26e+006	22

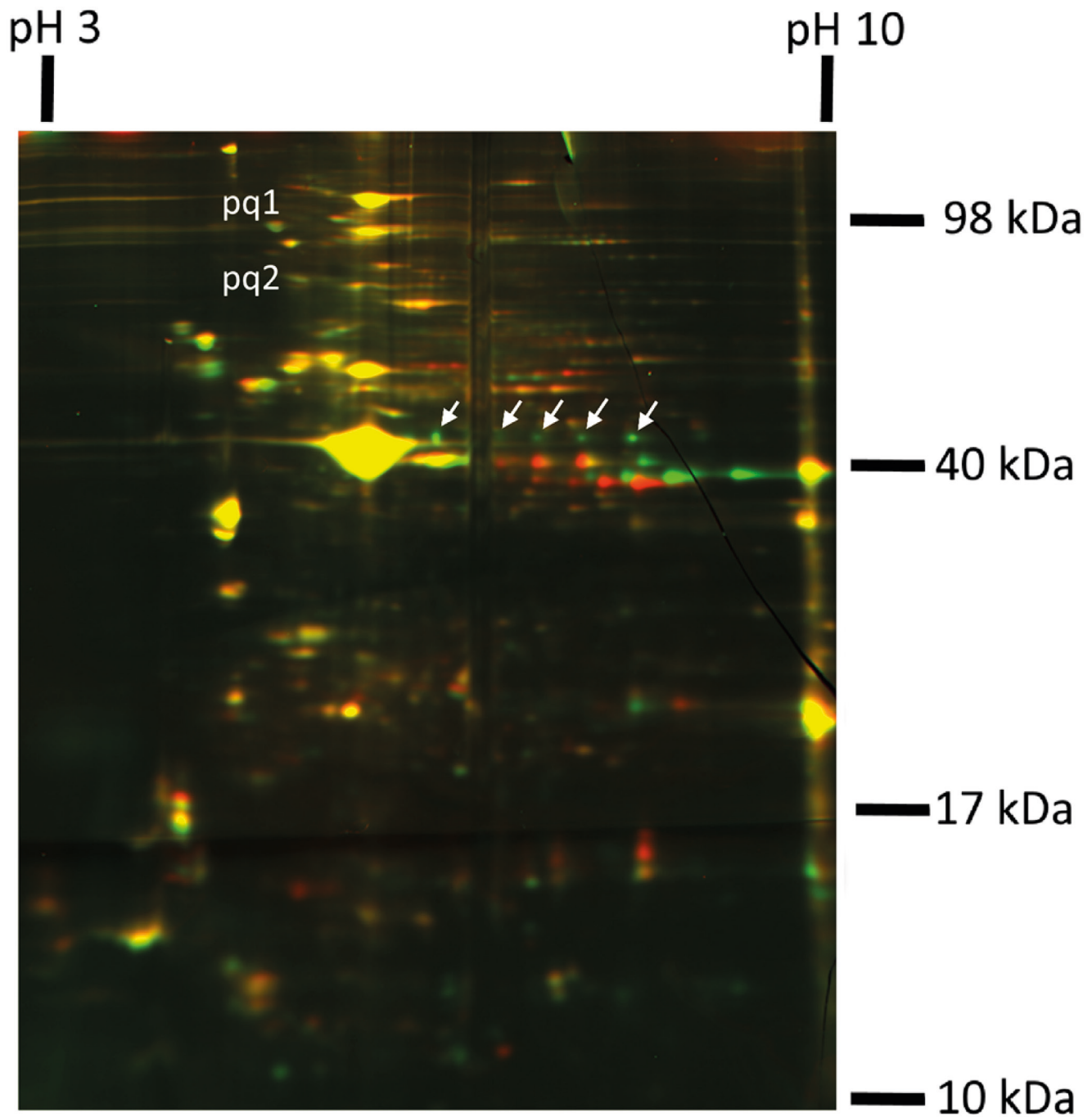
Table of identified unique peptide for pq1

Peptide sequence	Peptide MW (Da)	Number of spectra	Score	b/y series
(R)ELISNASDALDKIR(Y)	1544.8279	1	6.44	b <sub>2</sub> /y <sub>3</sub> -y <sub>6</sub> -y <sub>8</sub> -y <sub>9</sub> -y <sub>10</sub> -y <sub>12</sub>
(K)GWVSDDDLPLNVS(R)	1485.7544	4	16.25	b <sub>2</sub> -b <sub>4</sub> /y <sub>1</sub> -y <sub>2</sub> -y <sub>3</sub> -y <sub>6</sub> -y <sub>7</sub> -y <sub>8</sub> -y <sub>9</sub> -y <sub>11</sub> -y <sub>12</sub>
(K)SILFVPK(T)	803.5026	2	10.34	b <sub>2</sub> -b <sub>3</sub> /y <sub>1</sub> -y <sub>2</sub> -y <sub>3</sub> -y <sub>4</sub> -y <sub>5</sub>
(R)SSGTLEMSTIK(I)	1153.5769	3	9.93	b <sub>2</sub> /y <sub>2</sub> -y <sub>7</sub> -y <sub>9</sub>
(K)TLEINPR(H)	842.473	1	5.45	b <sub>2</sub> /y <sub>1</sub> -y <sub>2</sub> -y <sub>3</sub>
(R)TLQAKEEDLK(L)	1174.6314	1	4.95	y <sub>1</sub> -y <sub>4</sub> -y <sub>8</sub>
(R)YITFLR(V)	812.4665	2	5.80	b <sub>2</sub> /y <sub>3</sub> -y <sub>4</sub> -y <sub>5</sub>
(K)YLTFIR(G)	812.4665	2	5.80	b <sub>2</sub> /y <sub>3</sub> -y <sub>4</sub> -y <sub>5</sub>
(R)YLTFILR(T)	812.4665	2	5.80	b <sub>2</sub> /y <sub>3</sub> -y <sub>4</sub> -y <sub>5</sub>

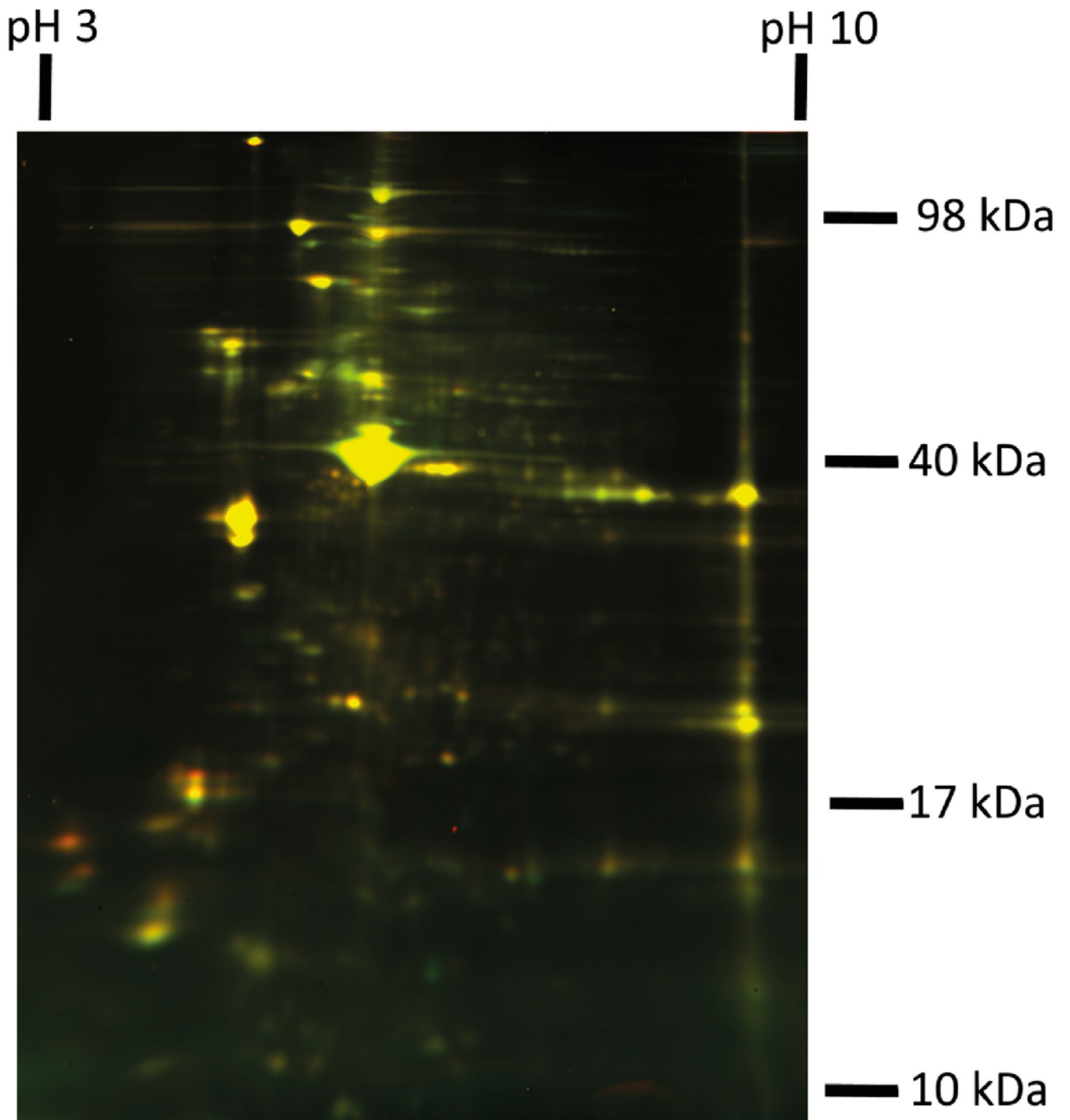


**SM 4 — Electrophoregrams of interspecies and inter-body-parts 2D-DIGE-comparison**

**Fig. 1.** 2D-DIGE of total lysates of mamilliform glands from *L. obtusata* (red) and *L. saxatilis* (green). Pq1 and pq2 are the proteins discussed in the main text with significantly higher expression level in the total lysates of mamilliform glands. Arrows show at *L. obtusata*-specific mamilliform gland proteins marked on Fig. 1 (a) and discussed in the main text. As mentioned in the main text, other species-specific proteins (red or green dots) were described earlier in Maltseva et al., 2016 as house-keeping proteins, expressed throughout an organism.



**Fig. 2.** 2D-DIGE of total lysates of the basal part of a penis from *L. fabalis* (red) and *L. saxatilis* (green). Pq1 and pq2 are the proteins discussed in the main text with significantly higher expression level in the total lysates of mamilliform glands. Arrows show at *L. saxatilis/L. arcana*-specific proteins from the basal part of a penis marked on Fig. 1 (a) and discussed in the main text. As mentioned in the main text, other species-specific proteins (red or green dots) were described earlier in Maltseva et al., 2016 as house-keeping proteins, expressed throughout an organism.



**Fig. 3.** 2D-DIGE of total lysates of the mamilliform glands from *L. arcana* (red) and *L. saxatilis* (green). The electrophoregram illustrates the absence of species-specific proteins in mamilliform gland proteomes in comparison between *L. saxatilis* and *L. arcana*.

### SM 5 — The discussion of possible mechanisms of gametic isolation in *Littorina*

For more detailed information on gametic isolation mechanisms in invertebrates see Lobov et al., 2019.

Gametic incompatibility is based on species-specific molecular interactions that prevent heterospecific fertilization. Gamete interactions in different taxa involve generally similar processes, but they often employ non-homologous molecules (the identification of their molecular functions is often a challenge). In externally fertilizing organisms gamete recognition includes only sperm and egg molecules. Apposite to them, gamete interactions in internal fertilizers (e.g. *Littorina*) are more complex: the basic stages of gamete recognition (chemotaxis of spermatozoa to an egg; induction of acrosome reaction and local destruction of egg envelopes by acrosomal proteins; egg and sperm membranes interaction and fusion) are combined with male-female interactions.

We suspect, that *Littorina* penial proteins (described in the main text) are involved indirectly to sperm-egg interactions, and through this they might be involved to reproductive isolation.

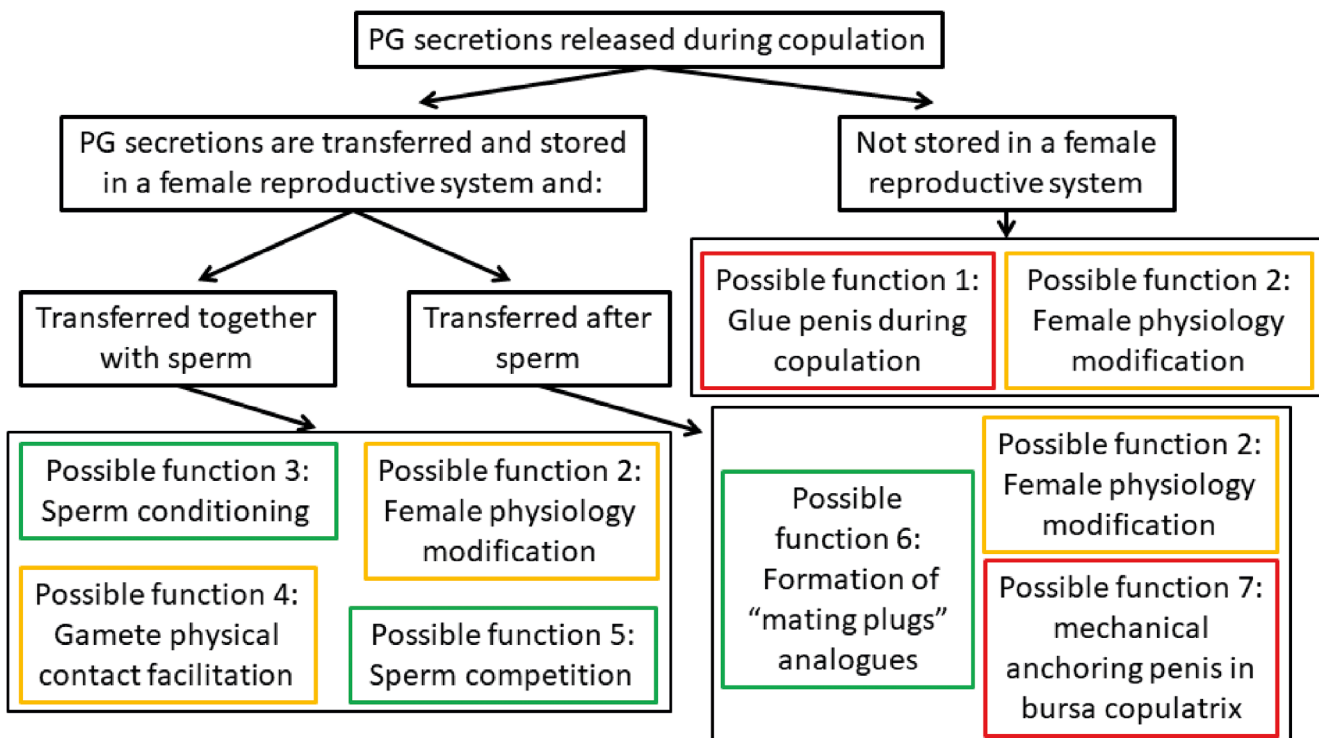
**Assumption 1:** if secretions of mamilliform glands or other penial glands are not entered to female copulatrix.

Possible function 1: Glueing a penis during copulation. This function of mamilliform gland secretions is aimed for keeping penis within female reproductive ducts during the copulation. It was firstly suggested in early studies and not disputed since then (Linke 1933; Bingham 1972; Reid 1989; Buckland-Nicks & Worthen, 1992).

Possible function 2: Female physiology modification. Numerous proteins transferred during copulation have strong effects on a female behavior and physiology (e.g. Avila et al., 2011); proteins of penial glands secretions might belong to this functional groups bringing some signal for a female organism.

**Assumption 2:** secretions of penial glands are mixed with sperm during copulation. In this case, possible functions of penial gland proteins are rather similar to proteins of seminal fluid, which are well studied in insects (e.g. Avila et al., 2011, 2016).

Possible function 3: sperm conditioning. In internally fertilizing organisms the state of spermatozoa is regulated by the components of seminal fluid and female reproductive tracts. According to this, proteins of penial gland being mixed with sperm can influence *Littorina* sperm state.



**Fig. 1.** Possible functions of proteins, secreted by penial glands (PG). In black frames there are presumable protein functions; in red frames — possible functions with no relation to reproductive isolation; in yellow frames — possible functions potentially connected with reproductive isolation; in green frames — possible functions directly related to reproductive isolation.



Possible function 4: gamete physical contact facilitation. Some proteins of seminal fluid are able to form filaments and complex three-dimensional structures, which mechanically facilitate fertilization. Similarly, PG proteins may be recruited to such complexes.

Possible function 5: sperm competition. In a case of polyandry, female mates with many males, and their sperm compete for an egg fertilization. Such competition is often regulated by seminal fluid proteins. Noteworthy, the results on *Littorina sitkana* (the *Neritrema* subgenus) showed no negative effect of the mamilliform gland secretions on a competitor's sperm (Buckland-Nicks & Worthen, 1992). Yet, this function cannot be excluded, as possible action of mamilliform gland secretions may occur, only when they are mixed with some female compounds or proteins of other penial glands.

**Assumption 3:** secretion of mamilliform glands or other penial glands is delivered to a female, but only after sperm.

Possible function 6: formation of “mating plugs” analogues. The production of physical barriers that prevent females remating is quite common in insects and other taxonomical groups, such as nematodes, arachnids, amphibians, mammals and cephalopods (Saad, 2018; Sutter, 2016). Nevertheless, it is rather unusual in gastropods. Moreover, the *Littorina* periwinkles are well studied at the morphological, ultrastructural, anatomical and physiological levels, and there are no signs of mating plugs. Thus, mating plugs formation is very unlikely scenario in the *Littorina* case.

Possible function 7: mechanical anchoring penis within a bursa copulatrix. Normal copulation in *Littorinas* takes 30–40 minutes, and the existence of special penis anchoring mechanisms is rather probable.