Revealing *Mytilus galloprovincialis* transcriptomic profiles during ontogeny

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ABSTRACT

Mediterranean mussels are a worldwide spread bivalve species with extraordinary biological success. One of the reasons of this success could be the reproduction strategy of bivalves, characterized by the presence of trochophore larvae. Larval development in bivalves has been a topic of raising interest in the scientific community but it deserves much more attention. The principal objective of this work was to study the transcriptomic profile of the ontogeny of *Mytilus galloprovincialis* analyzing the gene expression in different developmental stages, from oocytes to juveniles. For this purpose, after conducting a 454 sequencing of the transcriptomes of mussel hemocytes, adult tissues and larvae, a new DNA microarray was designed and developed.

The studied developmental stages: unfertilized oocytes, veliger, pediveliger, settled larvae and juveniles, showed very different transcriptomic profiles and clustered in groups defining their characteristic gene expression along ontogeny. Our results show that oocytes present a distinct and characteristic transcriptome. After metamorphosis, both settled larvae and juveniles showed a very similar transcriptome, with no enriched GO terms found between these two stages. This suggests: 1.- the progressive loss of RNA of maternal origin through larval development and 2.- the stabilization of the gene expression after settlement.

On the other hand during metamorphosis a specific profile of differentially expressed genes was found. These genes were related to processes such as differentiation and biosynthesis. Processes related to the immune response were strongly down regulated. These suggest a development commitment at the expense of other non-essential functions, which are temporary set aside. Immune genes such as antimicrobial peptides suffer a decreased expression during metamorphosis. In fact, we found that the oocytes which express a higher quantity of genes such as myticins are more likely to reach success of the offspring, compared to oocytes poor in such mRNAs, whose progeny died before reaching metamorphosis.

KEYWORDS: *Mytilus galloprovincialis*, larvae, ontogeny, development, oligo-microarray, Myticins

HIGHLIGHTS

- New *M. galloprovincialis* microarray represents its whole potential transcriptome.
- The study of mussel ontogeny highlights key processes of each developmental stage.
- Energy metabolism and defense are strongly regulated during metamorphosis.
- Myticins, the most important AMP in mussels, are detected through all development.
- The maternal immune transfer plays a key role in survival of the offspring.

1. INTRODUCTION

Mediterranean mussels (*Mytilus galloprovincialis*) are a worldwide spread bivalve species with an extraordinary biological success; they have indeed become one of the top 100 invasive species of all living organisms (Gardner *et al.*, 2016). The reproduction strategy of bivalves, characterized by the presence of trochophore larvae, is common to all lophotrochozoans, a group including Mollusca and Annelida among others (Halanych; 2004). In addition to exhibiting spiral cleavage and early cell fate determination, trochozoans typically undergo indirect development, which contributes to the most unique characteristics of their ontogeny. This larval development has presumably been maintained from the earliest Cambrian or even the Precambrian era, in which the fossil registry dates back molluscs (Ponder & Lindberg; 2008). Trochophore larvae are pelagic free-living and their development occurs until they undergo metamorphosis and then settle to a benthonic lifestyle. The length of this larval development is both species specific and dependent on environmental conditions. In the Mediterranean mussel it extends about one month.

A brief summary of mussel life cycle is depicted in figure 1. After male and female spawn, the embryo develops in 24 hours into trochophore larvae. The late trochophore is the phylotypic stage of molluscs, the point during life cycle of maximum similarity among the species of a phylum (Xu *et al.*, 2016). Trochophores use their two bands of cilia surrounding their bodies to swim and feed. At this stage the primordium of the shell starts to be secreted. The next day cilia develop into the velum, the most representative organ of the veliger stage, and the shell becomes evident. After 20 days, veligers lose their velum, larvae cannot swim anymore and the foot acquires importance. This indicates the entrance in the pediveliger stage, metamorphosis now happens and larvae settle when an appropriate substratum is found. Once metamorphosis is complete larvae resemble the adult form (Balseiro *et al.*, 2013) and the juveniles will attach to a substrate to grow and mature to the adult stage lifestyle.

The ontogeny of mollusks is a complex process only completed by a small percentage of the trochophore larvae. The main threats to the completion of the larval life cycle are predation, food limitation, adverse environmental conditions, including lack of appropriate substrate to settle, and pathogens (Eckman, 1996; Lambert *et al.*, 1998). Larval metamorphosis and settlement is a vital transition period associated with the evolution, differentiation and speciation of metazoans (Degnan and Degnan, 2010). Settlement can modify the larval dispersal capacity which is linked to the mortality risk, because animals that have correctly settled show increased survival. Therefore, settled larvae are important for the biological success of marine invertebrates (Williams and Degnan, 2009). In mussels immune capacities arise during mussel development as early as the trochophore stage but it is after metamorphosis that mussel larvae present an important switch in the gene expression pattern, reflecting the maturation of the immune system (Balseiro *et al.*, 2013).

The use of new genomic tools has increased the bivalve biology knowledge in topics such as ontogeny and evolution of trochozoans (Xu *et al.*, 2016), characterization of long non coding RNAs during development (Yu *et al.*, 2016), development of immune system (Balseiro *et al.*, 2013), hematopoiesis (Dyachuk, 2016) or trans-generational immune priming (Green *et al.*, 2016) and maternal transfer of immunity (Wang *et al.*, 2015). Nevertheless, molluscs' ontogeny is a limited area of knowledge that deserves much more attention. There are not gene markers for the different larvae stages, which could ease many research tasks and also the larvae rearing in hatcheries, a key step and a bottleneck for producers.

The principal objective of this work was to study the transcriptomic profile of the ontogeny of *M. galloprovincialis* analyzing the gene expression in different developmental stages, from oocytes to juveniles. For this study, a new DNA microarray comprising sequences of hemocytes, adult tissues and larvae was designed and developed.

To our knowledge, there are only two *M. galloprovincialis* microarrays previous to this design: Mytarray V1.1 (Venier *et al.*, 2006), a cDNA microarray used mainly to study pollution effects on mussel (Dondero *et al.*, 2010; Maria *et al.*, 2016); and *M. galloprovincialis* Immunochip (Venier *et al.*, 2011). This is the first microarray which contains probes from several adult tissues, including hemocytes, and also from different developmental stages.

The microarray platform and the microarray gene expression data can be found in the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>): GSE104153.

2. MATERIALS & METHODS

2.1 Sequence assembly, annotation and microarray design

A total of 1,325,571 raw reads from *M. galloprovincialis* were collected from sequencing of hemocytes, other adult tissues and also larvae from different developmental stages. Table 1 details the number of sequences by origin, assembly and annotation criteria. Briefly, a total of 1,325,571 reads have been obtained. After removing low-quality sequences and filtering for adaptors and primers reads were assembled through MIRA3 (default parameters). Assembly produced a total of 103,995 transcripts. A preliminary annotation was obtained for 43,218 contigs (41.5%) through BLASTx and BLASTn similarity searches conducted against several protein databases (e-value 10^{-5}). Alignments with an e-value threshold of 10^{-3} and 10^{-5} were considered significant for protein and nucleotide databases, respectively. Details on the annotation are reported in S1 File.

All databases used for the annotation step were considered for DNA microarray platform design. Putative sense-strand orientation was inferred from the matching protein-coding gene in reference data bases. For 1,803 contigs that showed ambiguous orientation two probes with opposite orientations (sense and antisense) were designed. For the remaining 41,415 contigs with putatively unambiguous orientation a single (sense) probe was designed. Since the microarray format could accommodate approximately 60,000 probes, the longer (bp) non-annotated contigs were included in the microarray design. In total 7,488 non-annotated contigs were added, and for each of them, two probes with opposite orientation (sense and antisense) were designed. Probe design was carried out using the Agilent eArray interface (<https://earray.chem.agilent.com/earray/>), which applies proprietary prediction algorithms to design 60-mer oligoprobes. A total of 59,971 out of 59,997 probes were successfully obtained, representing 50,680 different *M. galloprovincialis* contigs. S2 File provides the sequences of the contigs to design the probes and the probes of the *M. galloprovincialis* DNA microarray platform. Microarrays were synthesized *in situ* using the Agilent ink-jet technology with an 8×60 K format. Each array included default positive and negative controls. Probe sequences and other details on the microarray platform can be found in the GEO database under accession number GSE104153.

2.2 Larvae rearing, monitoring and sampling

Mature mussels were obtained from a mussel farm in Vigo (NW Spain) during the spawning season. Mussels spawning and larval rearing was performed as detailed in Balseiro *et al.*, 2013. Larvae and juveniles development was followed under a Nikon Eclipse 80i light microscope and photographed with a DXM1200 digital camera. Images were edited with the software NIS-Elements D 2.310. A fraction of oocyte, trochophore, veliger and pediveliger samples was prepared for scanning electronic microscopy (SEM). Samples were pre-fixed for 2 hours at 4°C with 4% glutaraldehyde in cacodylate buffer (0.1M, pH 7.2) and then washed twice for 3 hours in cacodylate buffer. Samples were further processed for SEM by the electronic microscopy service in the CACTI facilities

of the University of Vigo. Briefly, samples were post-fixed with OsO₄ 1% in cacodylate buffer (0.1M, pH 7.2) for 2 hours at 4°C and then washed twice for 3 hours in cacodylate buffer. Next, samples were mounted in the filter with the suitable pore size and dehydrated in ascending series of acetone washes (30%, 50%, 70%, 80%, 90%, 100%), 30 minutes at 4°C per wash, repeating the last 100% acetone step. Then, samples were critical-point dried using CO₂ (Baltec CPD030) and finally mount in stubs with carbon adhesive and a gold coating (Emitech K550X). Imaging of the larvae was achieved using a Scanning Electron Microscope FEI Quanta 200.

For the microarray hybridization five biological replicates were taken at each developmental stage: unfertilized oocytes, trochophore (1 day post fertilization; dpf), veliger (3 dpf) and pediveliger (20dpf) larvae, settled larvae (25dpf) and juveniles (30dpf).

Larvae were centrifuged at 3000 g for 10 minutes at 4°C. The pellet was resuspended in 250 µl of TRIzol (Invitrogen). Total RNA isolation was conducted following the manufacturer's specifications in combination with the RNeasy Mini kit (Qiagen) for RNA purification after DNase I treatment. Next, the concentration and purity of the RNA were measured using a *NanoDrop ND1000* spectrophotometer. Finally, RNA integrity was tested on an Agilent 2100 Bioanalyzer (Agilent Technologies). Only the samples with high RNA quality and quantity were used for labeling and hybridization, 3 to 5 biological replicates were used for each developmental stage and trochophore samples were discarded due to the low RNA integrity.

2.3 Cy3 labeling

Sample labeling and hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol. Briefly, 100 ng of RNA from each RNA sample was amplified and labeled with Cy3 using the Low Input Quick Amp labeling kit (Agilent Technologies), according to the manufacturer's instructions. Each sample included a mixture of 10 different viral poly-acetylated RNAs (Agilent Spike-In Mix) before amplification and labeling so the microarray analysis work-flow could be assessed. Qiagen RNeasy mini spin columns were used to purify amplified RNA. Finally, amplification and dye incorporation rates were verified using a *NanoDrop ND1000* spectrophotometer. These values should be between 200 and 500 ng/µL (RNA concentration) and between 20 and 50 pmol/µg aRNA (dye incorporation).

2.4 Microarray hybridization

Cy3 labeled RNA (600 ng) was fragmented with 5 µl of 10X Blocking Agent and 1 µl of 25X Fragmentation Buffer at 60°C for 30 min. Finally, 55 µl of 2X GE Hybridization buffer was added to dilute the fragmented RNA. The eight spaces of the gasket slide were filled with 40 µl of the correspondent hybridization solution and then assembled on the microarray slide (each slide contained eight arrays). Slides were incubated for 17 h at 65°C in an Agilent Hybridization Oven, subsequently removed from the hybridization chamber, quickly submerged in GE Wash Buffer 1 to disassemble the slides and then

washed in GE Wash Buffer 1 for 1 minute followed by one additional wash in pre-warmed (37°C) GE Wash Buffer 2.

Hybridized slides were scanned at 5 µm resolution using an Agilent G2565BA DNA microarray scanner (feature extractor barcodes available in File S3). Default settings were modified to scan the same slide at two different sensitivity levels (XDR Hi 100% and XDR Lo 10%). The two linked images generated were analyzed together, the data were extracted, and the background was subtracted using the standard procedures in the Agilent Feature Extraction Software version 9.5.1. The software returned a series of spot quality measures to evaluate the goodness and the reliability of the spot intensity estimates. After ensuring that all of the microarrays passed the quality tests (File S4), control features (positive, negative, etc.), except for SpikeIn Viral RNAs, were excluded from subsequent analyses. SpikeIn control intensities were used as internal controls and were expected to be uniform across the experiments of a given dataset.

2.5 Microarray statistical analysis

The GeneSpring software (Agilent) was used to normalize and analyze the microarray fluorescence data. A t-test ($p < 0.01$) with a Benjamini-Hochberg multiple testing correction was carried out in filtered raw data (20 - 90th percentile), to identify differentially expressed genes. The t-test was used to find the genes that were expressed significantly different among the developmental stages the oocytes, larval stages and also juveniles. Genes with a fold change between ± 1.5 were not used for further investigation. Additionally, an ANOVA ($p < 0.01$) was performed to find genes that were significantly regulated throughout the life cycle up to a mature juvenile stage. The post-hoc test used was Tukey HSD and the Benjamini-Hochberg multiple testing correction was also applied.

Clustering of the analyzed samples was performed with AutoSOME (Newman and Cooper, 2010). Clustering of myticins expression was performed in GeneSpring using k-means grouping. To effectively separate the myticins by their similar expression profile 5 groups were chosen, eventually 2 of them were fused again due to their high resemblance in their expression pattern.

2.6 GO terms and enrichment analysis

After statistical analysis, blast2GO software (Conesa *et al.*, 2005) was used to assign GO terms (Ashburner *et al.*, 2000) to the significantly expressed genes through development. Default values (annotation cutoff=55, GOweight=5) in blast2GO were used to perform the analysis.

The enrichment analyses were made with the total microarray information as the reference set and the list of differentially expressed genes of each developmental stage as the test sets. Then, Fisher's exact test was run: a one-tailed test, removing double IDs with a false discovery rate (FDR) cut-off of 0.01. In figure 4 only the most specific terms were represented.

2.7 qPCR analysis of oocytes

Mussel spawning was performed as previously described and eight families were maintained until larvae settled or died. A fraction of the spawned oocytes of each family were collected and concentrated using a nylon mesh and centrifuged. The pellet was resuspended in 500 µl of Trizol (Invitrogen). Samples were grouped as good or bad oocytes depending on the development of the offspring: if larvae reached the pediveliger stage and settled, oocytes were classified as good (families 45, 46, 48 and 49); otherwise oocytes were classified as bad (families: 47, 50, 51 and 52).

Total RNA extraction of oocytes was performed as described above and cDNA was synthesized with 1 µg of totalRNA using SuperScript™ III Reverse Transcriptase (Invitrogen) following the manufacturer's protocol. In each group the sample with the worst RNA quality were discarded (49 and 50). We finally kept 3 good oocytes samples and 3 bad oocytes samples for qPCR analysis.

Specific PCR primers for Myticin C, Myticin B, Mytimycin and C1q (Balseiro *et al.*, 2013) were prepared. Real-time quantitative PCR was performed in the 7300 Real Time PCR System (Applied Biosystems). One microliter of fivefold-diluted cDNA template was mixed with 0.5 µl of each primer (10 µM) and 12.5 µl of SYBR Green PCR master mix (Applied Biosystems) in a final volume of 25 µl. The standard cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. All reactions were performed as technical triplicates, and an analysis of melting curves was performed in each reaction. The relative expression levels of the genes were normalized using 18S as a reference gene following the Pfaffl method (Pfaffl, 2001) and standardized to the mean of the bad oocytes normalized expression to calculate fold changes. Prior to the statistical analysis a log 2 transformation of the data was performed (Rieu and Powers, 2009). A t-test for each of the studied genes was performed to evaluate differences in expression between good and bad oocytes. Being the “good oocytes” those whose offspring underwent metamorphosis and the “bad oocytes” those whose progeny died before reaching metamorphosis.

3. RESULTS & DISCUSSION

3.1 Larval development

The different *M. galloprovincialis* developmental stages were observed and photographed with a light microscope. Also, to illustrate the sampling of the larval stages, a subsample of each sample was prepared for SEM. Figure 1 shows a schematic representation of the mussel ontogeny with optic and SEM photographs of the gametes and the different larval stages in mussel: trochophore, veliger and pediveliger larvae. Ontogeny of bivalves has been studied mainly with optical microscopy (Loosanoff, Davies & Chanley, 1966). Electron microscopy approaches, mainly SEM, to characterize embryonic and larval development began at the end of last century and are still a field of interest, especially to study shell differentiation and its abnormalities (Schönitzer and Weiss, 2007; Aranda-Burgos et al., 2014; Balbi et al., 2016 and 2017).

The SEM images (Fig 1b) show the vitelline coat spikes of oocytes, a structure involved in the oocyte-sperm interaction (Focarelli *et al.*, 1991). Figure 1 b.B and b.C show the spermatozoa, with their characteristic pointed acrosome. We also noticed that the sperm always showed a grooved tail, something that to the best of our knowledge, has not been described before. Regarding the trochophore larvae, the two characteristic ciliary bands can be clearly distinguished both in the light microscope image (Fig 1a) and in the SEM image (Fig 1b.D). Figures 1 b.E and b.F show how the trochophore larvae develop the shell and how the cilia progressively grow to form the velum. The velum is a characteristic organ of veliger larvae whose functions are feeding and movement (Fig 1a, b.G and b.H). In the SEM images D to I a globular structure in the cilia of trochophore, veliger and early pediveliger larvae can also be clearly observed. To the best of our knowledge these structures had never been described in bivalve larvae and they seem to be lost after metamorphosis (Fig 1b.J and b.K). Consequently, they could have a specific function in the early mussel larvae feeding and/or movement. Prior to metamorphosis the velum loses importance at the same time that the foot acquires it. The foot can be seen retracted inside the shell in the light microscope picture of the pediveliger larvae. After metamorphosis the larvae body shape resembles that of the adult mussels as it is observed in the light microscope pictures. It is worth to note that the foot is blurred in figure 1a because the settled larvae showed very active movement of the foot to settle again after being sampled.

3.2 Assembly, annotation and microarray hybridization

A summary of the sequence origin, assembly and annotation results is shown in table 1. From the total 1,325,571 reads from *M. galloprovincialis*, the MIRA3 assembler was able to assemble 103,067 contigs. The putative identities of these sequences were obtained by Blast in protein and nucleotide databases. One probe for annotated transcript with known orientation was designed to construct a high-density oligo-DNA microarray, while two probes with both orientations were designed for contigs with ambiguous orientation or no annotation (Table 1). A total of 59,971 probes representing 50,706 unique transcripts were created using the Agilent eArray interface (<https://earray.chem.agilent.com/earray/>).

A total of 22 microarray experiments were successfully performed (File S3). The upper and lower fluorescence values were erased from the raw data (20 - 90th percentile) in all of the experiments, and only robust fluorescence values were used to analyze the expression and function of these genes.

3.3 Gene expression profile during ontogeny

To select genes that were significantly regulated along the development, we performed a t-test to find genes that were significantly different ($p < 0.01$) to the oocytes or the juvenile stage and the rest of the samples as shown in figure 2. The t-test yielded the identification of a total summatory of 78,921 differentially expressed genes (DEGs) between the different larval stages with regard to oocytes and 30,648 DEGs with regard to juveniles (30,728 and 25,528 non-redundant DEGs respectively). We considered these two comparisons because oocytes are haploid cells, and the most different cell type when compared to somatic cells, and juveniles are the most advanced developmental stage, with a similar behavior to adult mussels (Balseiro *et al.*, 2013).

The distribution of the DEGs using oocytes as a reference/control (Fig 2A) suggests the presence of RNA of maternal origin which is progressively lost. At the same time, developing larvae gradually increase the expression of their own RNA. Opposing to somatic cells, RNA and proteins in oocytes do not undergo a rapid degradation. RNA and proteins accumulated in the oocyte cytoplasm support the early phases of larval development (Gandolfi and Gandolfi; 2001). Maternal investment in protection of progeny has been previously detected in molluscs (Hathaway *et al.*, 2010). This is possible due to mechanisms such as cytoplasmic regulation of the polyA tail, RNA localization and protein phosphorylation to avoid the RNA and protein degradation in oocytes (Gandolfi and Gandolfi; 2001).

Although some other factors, such as epigenetic modulation could be involved (Riviere *et al.*, 2017), these results are in agreement and further support the scarce works on maternal immune transfer in molluscs (Yue *et al.*, 2013; Wang *et al.*, 2015). This is a field barely explored which, however, could improve the management of bivalves broodstock through the detection of the parental lines with the best immune background. In fish aquaculture it is common that the parental lines are well established and carefully maintained. A similar strategy in bivalves to find disease-resistant animals throughout their complete life cycle could be implemented. In oysters (*C. gigas*) resistant animals to OsHV-1 have been selected for breeding and culture (Dégremont *et al.*, 2015). Further studies have demonstrated that these resistant oysters are more susceptible to *Vibrio* infections (Azéma *et al.*, 2015), highlighting the importance of a careful selection before the election of the parental lines in hatcheries.

The differences among the developmental stages in *M. galloprovincialis* are clearly illustrated in figure 3. The AutoSOME clustering shows 4 well defined groups depending on their particular transcriptomes along ontogeny: Oocytes, veligers, metamorphosing pediveliger larvae and post-metamorphosis stages (settled larvae and juveniles). This figure represents oocytes as the most diverse cell type clustering altogether, although with

less strength than larvae phases, this fact reflects the diversity of oocytes compared to the fixed transcriptomes of the different larval stages. The veliger larvae of mussel develop to pediveliger stage fast, in a period of time of about 15-25 days (Satuito, *et al.*, 1994). This could be the reason that one of the veliger samples clustered among the metamorphosing pediveliger larvae. This clustering indicates that V4 sample developed quite fast if compared to the other veliger replicates. Larvae in metamorphosis cluster altogether and finally mussel settled larvae and juveniles show very alike gene expression and a very tight clustering, indicating the high pairwise affinity of their transcriptomes.

The veliger stage, even with almost 15,000 DEGs, is the most similar larval phase compared to oocytes. This far resemblance fades out at the same time that the larvae develop. The highest number of DEGs (23,364) was found between juveniles and oocytes (Fig. 2A and 1B), the most divergent developmental stages. The most alike stages were settled larvae and juveniles with only 199 genes significantly expressed (Fig 2B) and not a single biological process found to be enriched between these two stages (see “enrichment analysis” section below). The comparison among the developmental stages with regard to juveniles also showed that the number of regulated genes along ontogeny is drastically reduced after fertilization and that metamorphosis is the most particular larval stage, with over 4,000 DEGs, which deserves further attention.

Metamorphosis is a key developmental phase regarding the physiological aspects of the ontogeny. During metamorphosis, larvae modify their body structure, their behavior and acquire immunocompetence (Balseiro *et al.*, 2013). But there is a clear drive of the physiological efforts towards development and saving energy in consuming tasks such as immune defense, as it happens with swimming or feeding (Rodriguez *et al.*, 1993). For example, Myticins, one of the most expressed genes in mussels and a key protein for immune defense (Pallavicini *et al.*, 2008; Moreira *et al.*, 2015) are clearly down-regulated during metamorphosis (see section 3.4 for details), being the larval stage with the maximum down-regulation of this defense gene. Only 12 out of 69 myticin genes are up-regulated during metamorphosis compared to oocytes and 1 out of 282 compared to juveniles.

The Venn diagrams illustrate the expression of the exclusive and common genes between the different developmental stages regarding to oocytes and juveniles (Fig. 4A and 4B respectively). These Venn diagrams show from another point of view the hypothesis of the inheritance of RNA of maternal origin and the resemblance among larval stages. The general transcription pattern regarding to oocytes (Fig. 4A) is a common group of 9,154 genes many of them related to cytoskeleton but also to development, cell cycle and proliferation (anaphase promoting complex, angiopoietin, apoptosis-related proteins, autophagy related proteins, cell division cycle proteins, cyclin, cyclin-dependent kinases, hematopoiesis-related proteins, growth factors, etc.) and, as usual, myticin, the most expressed AMP in mussels. Figure 4A also shows an increasing number of exclusive genes for each subsequent larval stage except for settled larvae due to its resemblance to the juvenile stage (veliger has 1,768 exclusive DEGs, metamorphosis 2,468, settled 1,102 and juveniles 2,545).

On the other hand, the analysis of the gene expression pattern when compared to juveniles (Fig. 4B) shows that now the number common genes is drastically reduced to 58; 23 of them are informatively annotated revealing a core of genes related to extracellular matrix, defense and regulation of transcription such as collagen or trichohyalin (both matrix-related proteins), chelonianin (a serine protease inhibitor), C1q (a highly diverse recognition molecule), TNF receptor SF member 19 (activates JNK and NF- κ B and promotes apoptosis), DEAH box polypeptide 37 or even-skipped homeobox 1 (related to transcription). Again, Myticin appears in the common group of DEGs. The oocyte transcriptome, with almost 20,000 exclusive DEGs with regard to juveniles, is very different to the ones of the larvae after fecundation with much fewer regulated transcripts (figure 4B). Moreover, the transcriptome of the metamorphosing larvae has 1,137 exclusive transcripts. Among these we can find 69 Myticin transcripts, all of them, but one, strongly downregulated with fold changes that range from -10 to -160.

3.4 Role of AMPs and hematopoiesis-related genes during ontogeny

Myticins are the most important AMPs in mussels. They are thought to be the responsible of the resilience of these animals and resistance to infections which affect to other bivalves in the same area (Romero *et al.*, 2014). Myticins are produced and stored by hemocytes and their expression is extremely high in these defense cells (Pallavicini *et al.*, 2008; Moreira *et al.*, 2015). The mature myticin peptides kill different microorganisms by disrupting their membranes when they are released in the intercellular space after hemocyte degranulation, or in phagolysosomes (Mitta *et al.*, 2000). Myticins have been detected in all the tissues of adult mussels because of their open circulating system. We have also proved that oocytes can produce them as well as all larval stages, but myticins have not been detected in sperm.

This oligo-microarray contains 431 myticin probes, to cover the three myticin forms (A, B and C) and also as much myticin C hypervariability as possible (Pallavicini *et al.*, 2008; Costa *et al.*, 2009). To study their specific expression profile along ontogeny we performed an ANOVA which resulted in 326 differentially expressed myticin transcripts. With this information it is possible to infer the timing of the gene expression of these transcripts during *M. galloprovincialis* development. Myticins are encoded by different genes and the mechanisms resulting in their extreme mRNA variability has not been elucidated. This could be the reason why the regulation of all these transcripts is very different. In any case, myticin expression can be grouped in 4 clusters as shown in figure 5 and table 2. The groups reveal that the great majority of myticins are up-regulated after metamorphosis (cluster3), the same as other AMPs such as defensins and mytilins (Table 2), and a small group of 22 myticin transcripts (cluster 2) increase their expression levels at the veliger stage. The three myticin forms (A, B and C) are essential for mussel biology but these different patterns could be indicative of different roles along ontogeny and life cycle. Myticin A is the most represented AMP in all the clusters, balanced with Myticin C, and myticin B is much underrepresented except for cluster 1 (79% of probes in cluster 1 correspond to myticin B). Cluster 1 is characterized by the low expression of myticins in the metamorphosis stage resembling the myticin expression profile in the

metamorphosis stage regarding to oocytes (Fig S1). These results are, again, a clear indication of the inhibition of the expression of immune genes during the metamorphosis, as every developmental stage shows high expression of myticins over the oocytes except for the metamorphosing larvae. This does not mean that these larvae are completely unprotected in case of infection, many proteins can be stored in granules (the myticins, for instance) to be used if necessary during the short period of the metamorphosis (Novoa et al., 2016).

AMPs, especially Myticin C, are expressed at high levels in hemocytes (Balseiro *et al.*, 2011). This could be indicative of the early differentiation of this cell type and the maturation of the immune system. Table 2 also shows the expression of some genes related to hematopoiesis. Hematopoiesis is a controversial topic in molluscs (Pila *et al.*, 2016; Dyachuk, 2016). The hematopoietic organ has been identified in gastropods and cephalopods decades ago (Jeong *et al.*, 1983; Cowden and Curtis, 1981). In bivalves there are some hypothesis and studies which point to different tissues in different species and developmental stages: gills in adult oysters (Jemaa *et al.*, 2014) and mantle edge in mussel larvae and adults (Balseiro *et al.*, 2013; Moreira et al, 2015). We have identified some genes related to hematopoiesis in the microarray which were differentially expressed with regard to oocytes: the hematopoietic cell specific Lyn substrate 1 (HCLS1), exclusively expressed in cells of lymphohemopoietic origin in humans (Kitamura et al, 1989; ten Hacken *et al.*, 2013); the hematopoietically expressed homeobox (HHEX), an essential regulator of embryogenesis and hematopoietic progenitor development in vertebrates (Soufi and Jayaraman 2008; Paz *et al.*, 2010); the runt-related transcription factor 1 (RUNX1), an indispensable transcription factor for the establishment and precise maintenance of hematopoiesis in vertebrates (Sood *et al.*, 2017; Ichikawa *et al.*, 2013) and the peroxidasin (PXDN), an early differentiation marker for hemocytes in *Drosophila* (Grigorian *et al.*, 2011). Table 2 shows that, except HCLS1, down-regulated during all ontogeny, all of them show a similar pattern of expression: a strong upregulation in the veliger stage to decay and stabilize the rest of development. Hemocytes have many homeostatic functions in addition to their defense roles (Fisher, 1986). As larvae grow they need very soon a functional circulatory system to carry out basic physiological tasks: engulf and digest food particles, transport and release nutrients to hemolymph and cells, excretion of unwanted particles through feces and/or mantle cavity; tissue and shell repair (Donaghy *et al.*, 2009). This could be the explanation that genes such as HHEX, RUNX1 or PXDN are so highly expressed in veligers. In adult mussels (Moreira et al., 2015) these genes were previously detected in hemocytes, muscle, mantle and gill, but all of them showed different pattern of expression by tissue (details on the expression of these genes in File S5). RUNX1 was barely detected in gills meanwhile hemocytes and muscle showed the maximum expression; HHEX was clearly overexpressed in hemocytes, and PXDN, was the only gene whose maximum expression was not detected in hemocytes, but in the mantle.

3.5 Enrichment analysis

The sequences of the regulated genes exclusive for each developmental stage (t-test, $p < 0.01$) were analyzed in a Gene Ontology (GO) approach to perform a Fisher's exact test and detect the Biological Processes (BP) significantly enriched in each stage of ontogeny. After using the blast2GO tool to reduce the enriched terms, the most specific terms (the highest level GO terms of a parental line) were presented in figure 6.

No enriched BP GO terms were found for settled larvae, this may be explained by the fact that its transcriptome is very similar to that of juveniles (it is the larval stage with less exclusive DEGs as shown in Fig. 6 A and B) and its exclusive genes have similar functions of these found in the global transcriptome. On the other hand, the rest of the developmental stages present several interesting BP characteristic of each phase of ontogeny.

Veliger larvae showed enriched BP GO terms related to mitosis, due to the fast cellular division in early developmental stages, and to the assembly and organization of the axoneme, the proteins which form the core of cilia in eukaryotic cells (Fig. 6). The velum is a characteristic organ of veliger larvae. Veligers use the cilia in the velum for both propulsion and feeding, key features for survival and dispersal towards settlement; in consequence this process shows up at this stage. As veliger larvae become competent to metamorphose, the velum and its connections with the body deteriorate and finally this organ is lost (Arkett, 1988).

Metamorphosing larvae showed many BP GO terms related to lipid metabolism and differentiation of adipocytes (Fig. 6: acyl-CoA delta11-, palmitoyl-CoA 9- and stearoyl-CoA 9-desaturase activity, monounsaturated fatty acid and long-chain fatty acid biosynthetic process, regulation of cholesterol esterification, brown fat and white fat cell differentiation). This could be indicative of the change from larvae to a juvenile stage as the vitellus ends and the larvae had to store and now use their own energy source (Gallager *et al.*, 1986). As larvae develop towards metamorphosis, feeding slows down (Arkett, 1988). Survival to the juvenile form is dependent on many biotic and abiotic factors; the most important of them related to the availability of energy reserves accumulated (Rodriguez *et al.*, 1993). This is in agreement with the fact that during metamorphosis the mortality of larvae usually increases (Helm and Bourne, 2004). Mortality was usually attributed to the fewer amount of lipids stored by larvae but, in many cases larvae with high content of energy reserves did not reach juvenile stages. A possible explanation could be the great amount of Myticin transcripts significantly downregulated during metamorphosis. As a consequence "killing of cells of other organism" and "defense response to fungus", both GO terms related to Myticins, appear in the BP enriched at this stage. As we have hypothesized before, this could be an indication of the great physiological effort that larvae have to undertake to metamorphose and settle and the redirection of energy to these developmental tasks in detriment of defense, raising the mortality rates as well.

Juveniles most represented BP was the ephrin signaling pathway with more than 5% of the transcripts regulated, most of them actin and myosin. This pathway is fundamental in

the guidance of migrating cells during development via cell surface receptors and ligands which interact with cytoskeletal structures within the cell (Dravis, 2010). This could mean that during the juvenile stage many cell types are completing their migration towards their target position in the organism. It stands out since juveniles are almost an adult stage and it is interesting that this process seems so important at this late point of development. Another BP significantly enriched in juveniles is the metabolism of carbohydrates (Fig. 6A: glutamate catabolic process to aspartate, aspartate biosynthetic process, aspartate catabolic process, glutamate catabolic process to 2-oxoglutarate, oxaloacetate metabolic process, glycerol biosynthetic process, 4-hydroxyproline catabolic process, L-kynurenine metabolic process). This could be an indication that, finally, the basic homeostatic processes are gaining importance in the physiology of the larvae.

Oocytes are the developmental stage with the most amount of enriched BP, as they are the most different cell type among all the samples. In figure 6B are represented the top 40 of 296 BP overrepresented in the oocytes transcriptome when compared to juveniles. There is one group of processes especially numerous in this analysis (representing 47% of all enriched BP): development, with GO terms related to stem cells, morphogenesis, differentiation, proliferation, growth, mitosis..., and also other less represented groups like gene expression and its regulation (13%) (mRNA, siRNA, piRNA, histone methylation and acetylation, transcription), defense (6%) (apoptosis, MAPK cascade, JNK cascade, TNF signaling, lectin) and DNA maintenance (3%) (DNA repair, telomere maintenance). These categories represent important processes related to growth highlighting once again the importance of the oocyte transcriptome quality for offspring developmental success.

3.6 Immune maternal transfer and offspring success

We reared 8 families until larvae underwent metamorphosis (good families: 45, 46, 48, 49) or until the larvae died before reaching metamorphosis (bad families: 47, 50, 51, 52). One of the things we have noticed is that the quantity of oocytes after spawning does not correlate with the success of the offspring. Indeed, three of the good families (45, 46, 49) had spawnings with the least amount of oocytes. On the other hand, in all of the bad families the quantity of oocytes in the spawning was big. Family 50 did not even had a successful fertilization and family 47 died the third day after fertilization, families 51 and 52 died 22 days after fertilization but no larvae reached metamorphosis. The D-larval rate for the good families ranged from 22% to 67% (mean 44.68%) and for the bad families from 0% to 22% (mean 11.11%).

Some studies related to oocyte quality and success of offspring in bivalves have been recently published for scallop (*P. maximus*) (Pauletto *et al.*, 2017), clam (*R. decussatus*) (De Sousa *et al.*, 2014) and oyster (*C. gigas*) (Corporeau *et al.*, 2012) following transcriptomic and proteomic approaches. These works proved that proteins such as chaperones, vitellogenins, or genes related to cell cycle control, stress or apoptosis, are key not only for fertilization but also for embryonic development and progeny success. In the particular case of *M. galloprovincialis*, AMPs and other immune-related genes seem

to be good markers for prediction of offspring success. As shown in figure 7 Myticin C, Myticin B, Mytimycin, Apextrin P and C1q domain-containing proteins expression were directly related to the success of offspring. All studied genes but Mytilin B showed a statistical difference between the good and bad oocytes RNA load of these immune related genes. The representation of the results is quite revealing, and although for Myticin B no statistical significance was obtained, a strong tendency is observed: oocytes with high expression levels of these genes lead to offspring that successfully reached metamorphosis. The high dispersion of the results of the good families, typical in wild animal samples and usually leading to no significant results, was not enough to hamper the importance of the maternal immune transfer.

These results highlight the importance of the role of maternal immune transfer in the mussel ontogeny. Maternal transfer has been studied in molluscs larvae as a part of the innate immune response. It was recently verified that molluscs oocytes possess significant antibacterial, lysozyme and agglutinating activities against pathogens; many of these immune factors have been identified also in embryos (Wang *et al.*, 2015). Trans-generational immune priming has been demonstrated in oysters thanks to this mechanism (Green *et al.*, 2016). Maternal transfer of immunity is an effort that the females do to benefit their offspring. This beneficial investment protects eggs and embryos from horizontal (environmental) and vertical (parental) transmission of pathogens, which eventually means an increased offspring survival.

4. CONCLUSIONS

In the present study we present the first oligo-microarray of *M. galloprovincialis* which includes sequences from all the main tissues, including stimulated hemocytes, oocytes and larval and juvenile stages. We have analyzed the transcriptome of 5 different developmental stages from unfertilized oocytes to the juvenile stage and found thousands of differentially expressed genes out of 59,971 probes, presumably representing the whole transcriptome of the Mediterranean mussel. This the first time the transcriptome of oocytes and four larval and juvenile stages have been studied as a whole. We have found significantly regulated biological processes of vital importance during ontogeny and a clear vision of the maternal RNA transfer to offspring and how it relates with the success in development. This oligo-microarray for *M. galloprovincialis* has proven to be useful in developmental expression analyses. And although nowadays NGSs are the chosen technology to analyze transcriptomes, this tool is a cost-effective and relevant technology for targeting specific research questions.

In summary, maternal RNA transfer seems to play a key role in development and it deserves further research. Additionally, proteomics and epigenetics in ontogeny are also interesting fields that should be investigated in the near future as well as the role of alternative regulatory strategies during larval development such as RNAi or small RNA.

AVAILABILITY OF SUPPORTING DATA

Microarray data are deposited in the public functional genomics data repository GEO: GSE104153

SUPPORTING INFORMATION

Figure S1

File S1: Annotation details

File S2: Sequences of the original contigs and microarray probes.

File S3: 22 pdf files with details of the quality control of each microarray experiment.

File S4: Samples successfully hybridized with their specific microarray code.

File S5: Gene expression of hematopoiesis-related transcripts in adult mussel tissues.

Table S1: Top 20 DEGs during ontogeny vs oocytes. **A.** veliger larvae and metamorphosis stage. **B.** settled larvae and juveniles.

Table S2: Top 20 DEGs during ontogeny vs juveniles. **A.** oocytes and veliger larvae. **B.** metamorphosis stage and settled larvae.

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AUTHORS' CONTRIBUTIONS

BN and AF conceived and designed the experiments. RM and PB prepared the samples. RM, MP and MM hybridized the microarrays. LB, MP and MM contributed with labeling and hybridization reagents and scanning machinery. PP performed the qPCRs. BN, AF and RM analyzed the data. RM wrote the paper. All authors read and approved the manuscript.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ETHICS STATEMENT

The Mediterranean mussel, *M. galloprovincialis*, is not considered as an endangered or protected species in any international species catalogue, including the CITES list (www.cites.org) and it is not included in the list of species regulated by the EC Directive 2010/63/EU. Therefore, no specific authorization is required to work on mussel samples.

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Table1. Description of the microarray design process

Sequence origin	
454 tissues and larval stages	878,235 raw reads
454 hemocytes	447,336 raw reads
Assembly	
Contigs	103,067
Annotation	
Annotated Contig	43,218
Microarray	
Sequences used to design probes	50,706
frame +	36,147
frame -	5,244
Ambiguous frame	9,290
TOTAL successfully designed probes	59,971
Transcripts represented in microarray	50,680

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Table2. Expression pattern of AMPs and hematopoiesis-related DEGs during ontogeny.

Description		FC Vel	FC Met	FC Sett	FC Juv
Mean Myticins					
% myticins cluster 1	Cluster 1	-4.67	-22.29	-7.34	1.10
6.45%	Myticin-A cluster 1	-3.12	-5.26	-6.28	-1.58
79.03%	Myticin-B cluster 1	-4.84	-22.95	-7.47	1.26
14.52%	Myticin-C cluster 1	-4.14	-28.92	-6.80	1.55
% myticins cluster 2	Cluster 2	14.18	8.60	4.21	3.73
59.09%	Myticin-A cluster 2	11.08	7.56	4.65	3.48
18.18%	Myticin-B cluster 2	33.48	17.47	6.61	8.70
22.73%	Myticin-C cluster 2	6.82	4.23	1.15	0.41
% myticins cluster 3	Cluster 3	-5.89	-5.71	6.08	12.01
55.00%	Myticin-A cluster 3	-5.60	-4.80	6.38	11.91
3.00%	Myticin-B cluster 3	-4.61	-6.41	3.76	7.38
42.00%	Myticin-C cluster 3	-6.36	-6.84	5.84	12.47
% myticins cluster 4	Cluster 4	2.87	0.51	9.06	19.34
54.76%	Myticin-A cluster 4	3.53	0.40	7.00	18.24
4.76%	Myticin-B cluster 4	0.94	-0.54	4.84	9.13
40.48%	Myticin-C cluster 4	2.24	0.78	12.35	22.01
Defensins					
ALL2_C48785_P	Big defensin	25.12	35.86	39.02	117.86
ALL2_REP_C17609_P	Defensin MGD-1	10.91	3.75	34.00	163.42
Mytilins					
ALL2_C46224_P	Mytilin-2	6.40	51.77	2523.56	1319.60
ALL2_REP_C23330_S	Mytilin-A	3.96	31.08	325.07	240.87
ALL2_REP_C28588_P	Mytilin-B	-2.04	4.57	25.33	17.60
Hematopoiesis-related					
ALL2_REP_C46219_P	HCL1	-3.08	-6.12	-4.19	-3.58
ALL2_C1043_P	HHEX	83.49	45.09	31.33	41.68
ALL2_C954_P	RUNX1	46.96	9.92	8.68	15.58
ALL2_C45361_S	PXDN	500.26	78.30	43.15	30.32

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Figure captions

Figure 1. Ontogeny of the *Mytilus galloprovincialis*. **a:** Schematic representation of mussel life cycle. Scale bar for the gametes and larval stages represent 50 μm . **b:** SEM images of gametes and larvae until metamorphosis. **A.** Oocyte; **B.** Spermatozoa; **C.** Head detail of spermatozoid; **D.** Trochophore larvae; **E.** Late trochophore larvae; **F.** See how the primordium of the shell is almost covering the larvae; **G.** Veliger larvae; **H.** Detail of the velum of veliger larvae, note the globular structures in in velum cilia; **I.** Metamorphosing larvae, see how the velum is about to be detached; **J.** Pediveliger larvae, see the successive layers of calcification of the shell; **K.** Detail of the mantle border of pediveliger larvae, note that the globular structures are almost lost.

Figure 2. Distribution of regulated genes during ontogeny. Statistically significant gene modulation is subdivided according to intensity (fold change) and sense (up and down-regulation). **A.** Fold changes are indicated using the oocytes as the reference. **B.** Fold changes are indicated using the juveniles as the reference

Figure 3. Clustering of the analyzed samples performed with AutoSOME. Pairwise affinity is defined as the extent to which two samples co-cluster (0 = samples never co-cluster; 1 = samples always co-cluster).

Figure 4. Venn diagram showing the common regulated genes among the four samples.
A. Using the oocytes as the reference. **B.** Using the juveniles as the reference

Figure 5. Normalized fluorescence values of the 326 mytacin probes in the microarray significantly detected by the ANOVA (**top**) and clustering by expression pattern (**bottom**).

Figure 6. Enrichment analysis. Representation of the BP associated to the exclusive d.e.g for each larval stage with regard to the reference set (all sequences in the microarray). **A.** Exclusive DEGs of each stage vs oocytes. **B.** Exclusive d.e.g of each stage vs juveniles.

B. Enriched BP

exclusive DEG vs juveniles

Figure 7. qPCR analysis of immune-related genes of oocytes from a single female which derived in good or bad families. Data are shown as a dot plot and the mean as a solid line. Asterisks show the statistical significance: *** p-value < 0.001; ** p-value < 0.01; * p-value < 0.05

Figure S1. Comparison of the mytacin grouping by the k-means clusters (ANOVA) and the differential expression in each developmental stage vs oocytes. A representation of the fold changes for the ANOVA clusters are also shown.