Unconventional model systems; Tardigrada, Nematoda and Brachiopoda as prospective candidates for investigation into the maternal-to-zygotic transition and early development.

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Abstract

The maternal-to-zygotic transition is a process in which embryos transition from their maternally deposited mRNAs and proteins, to the transcription and translation of their own genome. Maternal products deposited into the oocytes during oogenesis are essential in the early development of embryos. Many of them lay the groundwork for axis specification, germ layers and mitosis. Zygotic transcription is often dormant for several cleavages. At a certain point the maternal products start to degrade, and the zygotic genome is transcribed, translated and fully responsible for development. While much is known of the process in Ecdysozoan species such as *Drosophila melanogaster, Caenorhabditis elegans* and vertebrate species such *Xenopus laevis* and *Xenopus tropicalis,* very little information exists outside these established model systems. The results of this study show that transcription of the genome is required for further development in the nematode *Pontonema vulgare* and the tardigrade *Hypsibius dujardini* after the fourth and fifth cleavage cycle respectively through inhibition of transcription. In addition, key maternal products can be detected in *Terebratalia transversa* early stages by way of in-situ hybridization. The results give an insight into the maternal-to-zygotic transition within *Pontonema vulgare* and *Hypsibius dujardini* as well as an insight into early development and maternal factors in *Terebratalia transversa*. These three species could all pose as valuable model systems in their own right as the phylogenetic position of both *Pontonema vulgare* and *Hypsibius dujardini* in the Ecdysozoa render them comparable to their Ecdysozoan relatives, and the *Terebratalia transversa* as a prominent representative of the lesser known Brachiopoda enables all three species good candidates for investigation into the maternal-to-zygotic transition and early development.

1 - Introduction

1.1 - The maternal-to-zygotic transition

Early embryo development is characterized by the maternally deposited products in the oocyte during oogenesis. The embryo will develop in the presence of these maternal deposits without the need for transcription of further mRNAs, up until a certain point.
At some stage in the early development however the embryo's own zygotic genes are activated, this is known as the zygotic genome activation (ZGA), whilst maternal mRNAs and proteins are gradually degraded, otherwise known as maternal degradation. The entire process is known as the maternal-to-zygotic transition (MZT). The event is subsequently divided into waves, usually across species it has been found one primary wave of maternal degradation followed by a secondary which often is concurrent with the minor wave of zygotic transcription. This is then followed by the major wave of zygotic transcription, where at this point all maternal products have been degraded. Tadros & Lipshitz defines the MZT as: "the period that begins with the elimination of maternal transcripts, continues through the production of zygotic transcripts and ends with the first major morphological requirement for zygotic transcripts in embryonic development." [1] The transition is vast, complex and highly divergent within all model and non-model species [2].

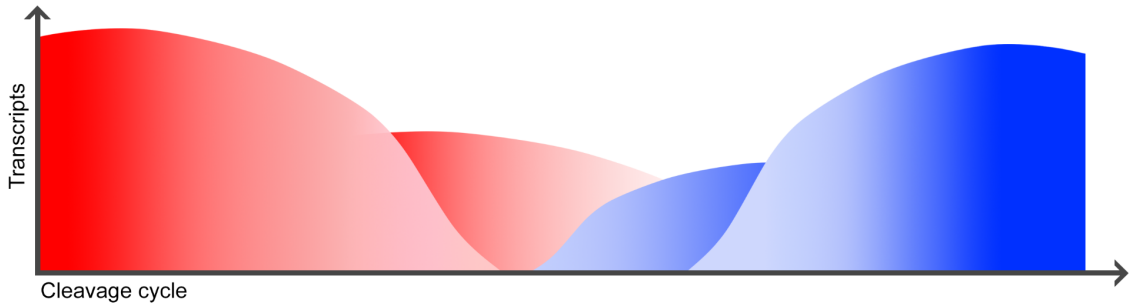

Figure 1.1.1 - The maternal-to-zygotic transition simplified
The red curves signify the two waves of maternal degradation. The blue curves represent the two waves of zygotic genome activation. The figure is adapted from [3].

A large amount of mRNAs and proteins are deposited into the oocytes of any species during oogenesis. Transcriptomic studies estimate that circa one third of the total protein coding transcriptome are deposited as mRNAs in *Caenorhabditis elegans* [4], [5] and mice [6]. In the fruit fly *Drosophila melanogaster* and the zebrafish *Danio rerio* an estimated

three-quarters are deposited [7]–[12]. The maternal products are present in early embryos in large quantities, and are essential for the embryonic development.

The onset of zygotic transcription is then linked to the decay of maternal products, however, zygotic transcripts are often not essential at the first sign of transcription, since transcripts may be detected considerably earlier than the minor wave of ZGA [13]–[15]. The timing of the event, and the interplay between maternal and zygotic products varies greatly across species. Note that the definition of the MZT by Tadros & Lipshitz [1] adds that the starting point of the MZT is egg activation. The time periods described below rather outline the period in which the transformation is at its most drastic, though they coincide in some cases.

1.1.1 - Oocyte-to-embryo transition

The oocyte-to-embryo transition (OET) is a process involving fertilization and maturation of oocytes to mitotically dividing embryos, triggered upon fertilization of oocytes [16]. Maternally deposited mRNAs and proteins will allow the egg to activate.

While the OET is not synonymous with the MZT, the two processes are highly intertwined, as they both rely solely on maternal products and may even coincide temporally as in mice where maternal degradation occurs post-meiosis [6], [17] or in *Drosophila* where some degradation occurs prior to fertilization [18], [19]. Even so, definitions vary among researchers and the definition of the MZT employed here in this thesis is the one provided by Tadros & Lipshitz [1] which defines the MZT's starting point as the egg activation, and not egg fertilization as some do [20]. The definitions therefore neatly separate the OET and MZT at the stage of egg activation.

1.1.2 - MZT timing in model organisms:

In *C. elegans* maternal degradation starts at the one-cell stage whilst the remainder of maternal transcripts are rapidly degraded soon after the 4-cell stage [4], [5], whilst zygotic transcripts can be detected as early as the four-cell stage [21], [22]. However, embryos cleared of RNA pol II subunit AMA-1, which is essential for transcription, exhibit no defects in division until the 28-cell stage [13]. At the same time, treatment with the transcriptional inhibitor and toxin alpha-amanitin, which binds the AMA-1 subunit of RNA pol II [23], shows that embryos may cleave until the 28-cell stage in the absence of zygotic transcripts [14]. Embryos cleared of subunit AMA-1 or treated with alpha-amanitin arrests the cleavage cycle following the 28-cell stage.

Similar observations have been made in other model systems such as *Drosophila melanogaster.* The primary wave of degradation in *Drosophila* does not even require fertilization of the egg, but most products decay rapidly following the 6th cleavage cycle [18], [19], [24]. A miniscule amount of zygotic transcription is detected as early as prior to the minor wave of ZGA, just 30 minutes post-fertilization, while the minor wave of the ZGA occurs at around the 7^{th}-8^{th} cleavage cycle [7]. The embryo cleaves in absence of ZGA until the thirteenth cycle by alpha-amanitin treatment [25].

In *Xenopus laevis* and *Xenopus tropicalis* early zygotic transcripts can be detected as early as the 2-cell stage [26] which coincides with the first wave of maternal degradation, where one third of the maternally deposited products are degraded [27]. The rate is consistent up until the minor wave of ZGA at the 64-cell stage [28], in which maternal products are rapidly degraded. Interestingly embryos injected with alpha-amanitin have been shown to develop up until the 15^{th} cleavage cycle showing no defects [29], after this point, cleavages were arrested.

In mice, the first wave of maternal degradation occurs post-meiosis and the second following fertilization of the oocyte. Fittingly, zygotic genome activation is detected following fertilization, and the minor wave occurs at the 2-cell stage in which maternal products are rapidly degraded [17], [30]. By the 4-cell stage maternal products are all but degraded coinciding with the major wave of ZGA [28], [31].

In the zebrafish the earliest zygotic transcripts can be detected after the 6^{th} cleavage cycle, while the majority of zygotic transcripts are transcribed following the 10^{th} cleavage cycle [32], [33]. As for degradation of maternal products about 60% are degraded between the fertilized egg and the 4^{th} cleavage cycle, the remainder are degraded coinciding with ZGA [11], [34], [35].

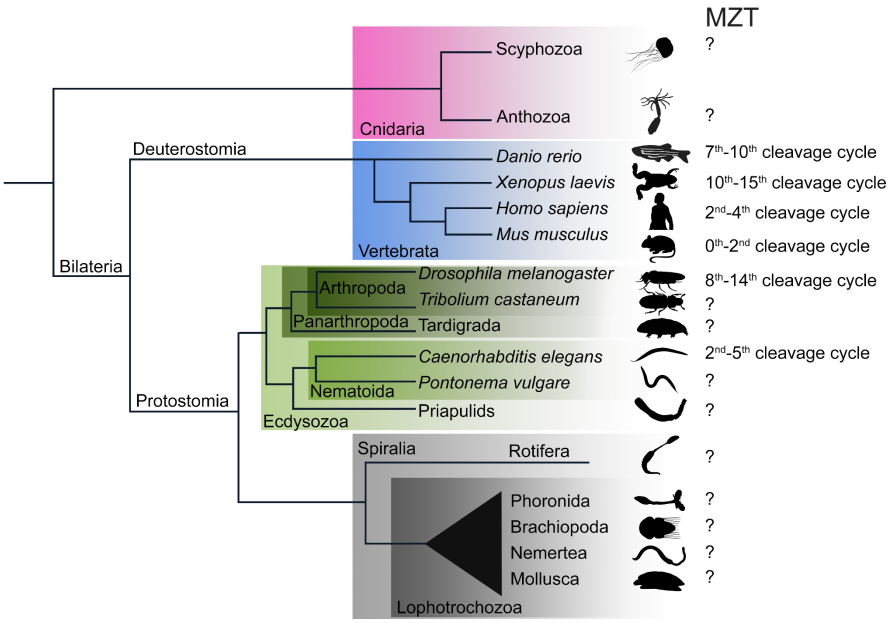

Figure 1.1.2 - The timing of the maternal-to-zygotic transition across select species

A tree showing the known timing of the MZT and the methodology used to determine it across select species of Ecdysozoa and Vertebrata while also highlighting Spiralia and Cnidaria. The timing of the MZT is shown in species where known. Note that the timing as described here does not entail the entirety of the process, as miniscule levels of zygotic transcription and maternal degradation may be detected earlier, the timing rather highlights when the majority of the MZT occurs. For the timing of the event in species as stated methodology include transcriptomics, microarray, chromatin and histone modifications, and inhibition of transcription.

1.1.3 - Models that explain ZGA activation

The onset of zygotic transcription is delayed as the maternal deposits direct development in the earliest stages. In broad strokes, the timing might be explained by: 1) factors needed for zygotic transcription are inactive or not present and 2) factors needed for transcription are present but inhibitors repress expression of the genome.

A major model for explaining the activation of ZGA is the notion that division cycles could regulate transcription through change in the nucleocytoplasmic (N:C) ratio model [36]. Through each division, the ratio of volume of genetic material to cytoplasmic volume increases, which may facilitate transcription. A maternal inhibitor could repress transcription at the earliest stages, but following division may be diluted in contrast to the genetic volume which increases. As such, transcription may start once the inhibitor is diluted sufficiently. This model has been supported in various experiments performed in *Xenopus*, in polyspermic embryos it was shown that ZGA occurs earlier than when fertilized by one single sperm [37], injection of plasmid DNA caused premature ZGA [38], increase in cytoplasmic volume by cleavage inhibitors caused a delayed ZGA [37] (fig1.1.3A). The model was however challenged when it was shown that haploid *Drosophila* embryos execute ZGA with expected

timing [39].

Another model explaining the activation of the zygotic genome is the maternal-clock. This model posits that egg fertilization or egg activation engages a cascade of reactions which serves as a finely tuned timer. The model was proposed when it was found that Cyclin A and Cyclin E1 in *Xenopus* were degraded independently of the N:C ratio [40]. Maternal deposits which would normally drive zygotic transcription may already be present, but inhibited by RNA-binding proteins (RBPs). The cascade may then serve to lift the inhibition of mRNAs, drive polyadenylation, translation and transport to the nucleus, leading to ZGA. A few such factors have been identified, such as Smaug in *Drosophila melanogaster* [8], [24], TFIID components in *Caenorhabditis elegans* [41] and several transcription factors in *Xenopus* [42], [43] (fig 1.1.3B)

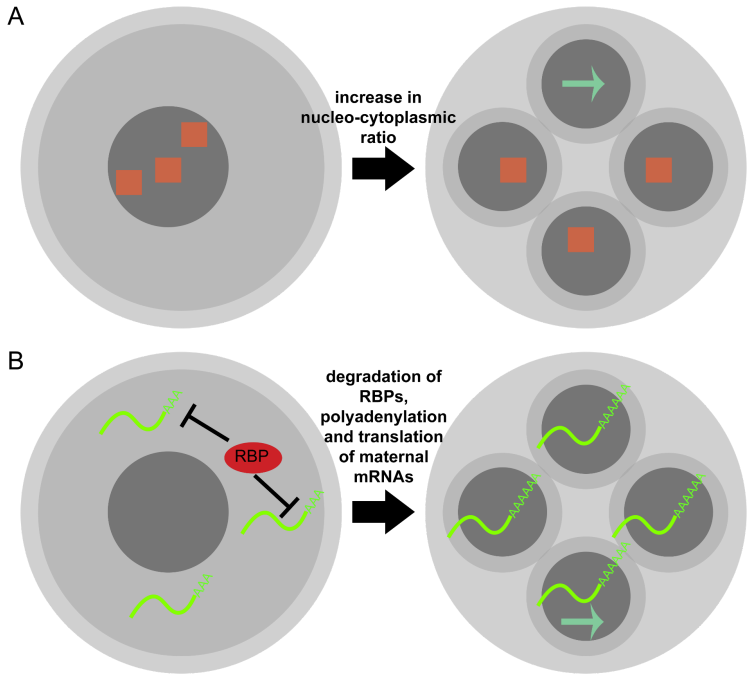

Figure 1.1.3 - Models that explain ZGA timing

A: Nucleocytoplasmic ratio model - an increase in the ratio of genetic material to cytoplasmic material may dilute repressors, shown as orange squares, and allow for transcription once diluted sufficiently.

B: Maternal clock model - a cascade starting at egg fertilization or egg activation eventually leads to either degradation of RNA-binding proteins (RBPs) shown in red, polyadenylation, translation or nuclear transport of mRNAs leads to zygotic transcription.

These models are just part of the machinery involved in activation of zygotic transcripts. They have both been proven to be true, but that does not make them mutually exclusive.

Both may co-exist as models for explaining the onset of ZGA and may just be a fraction of the total explanation for the specific timing.

1.2 - Maternal products and embryo development

All bilaterian bodies are made up of two axes, the anterior-posterior (AP) and the dorsal-ventral (DV). The AP-DV identity of cells within an embryo later gives rise to specific organs, limbs and specific tissues . During early embryonic development the axes of the anterior-posterior and dorsal-ventral are specified. Axis-defining mRNAs and proteins are often a direct result of gradients laid down by maternal products [44]–[46] or zygotically activated genes [47]. The formation of these axes lay the groundwork for the body plan of the adult individual and are therefore essential. The pathways leading to these specifications are often conserved, but some vary.

The sog and dpp genes pattern the DV axis as shown in the fruit fly *Drosophila melanogaster* [48], the pathway is highly conserved across species but also varies in effect [48], comparatively in *Xenopus*. The fruit fly derived sog (short gastrulation) and the *Xenopus* derived chordin are similar, but while *Xenopus* chordin serves to dorsalize [49], Sog is a ventralizing factor that creates a DV boundary by transporting dpp to the dorsal side [47]. Sog and dpp homologs have also been found in other animals, such as the beetle *Tribolium castaneum* in which the pathway is somewhat less complex [47]. The sog gene is activated zygotically and is not a maternal factor deposited to the oocyte, but is however detected in early development of embryos [50].

Hunchback is an anterior specifying factor in *Drosophila*, expressed uniformly but translationally inhibited in the posterior by nanos [51]. Homologues have been found in other animals, showing a highly conserved function in other insects [52], [53] in the beetle *T. castaneum* [54] and in the crustacean *Artemia franciscana* [55]. However there is evidence to support that the conserved function arose in Arthropods, and may not be universal for Ecdysozoans [56].

Toll is a maternal ventralizing factor and the receptor for the ligand spätzle in the fruit fly. Although toll is distributed throughout the membrane of early *Drosophila* embryos, its activation is highest at the ventral region due to the ligand spätzle being activated in this region. The binding of the ligand to the toll receptor allows the transcription factor dorsal to enter nuclei ventrally [57]. Embryos lacking Toll have been shown to be dorsalized, as dorsal does not enter any nuclei [46].

The canonical Wnt/beta-catenin pathway is yet another axis specifying system. In *Xenopus,* Wnts are deposited maternally in embryos. The Wnts are ligands to frizzled receptors, which when bound will allow beta-catenin to serve as a transcription factor. The pathway serves to dorsalize embryos and specify the posterior in larvae [58]. In the absence of Frizzled-7 receptor, which is expressed in the animal pole and dorsal region post-gastrulation [59], embryos were shown to ventralize [60], while injection of Beta-catenin to the ventral region of 32-cell stage *Xenopus* embryos eventually leads to twinned embryos [61]. The canonical pathway has been observed in many other animals, including the zebrafish *Danio rerio* [62].

Inhibition of BMP by secretions of the Spemann organizer in *Xenopus*, which is in part induced dorsally by Beta-catenin, creates a DV gradient of BMP and its downstream targets. This is similar to the process of inhibition of BMP in the zebrafish by the shield which is functionally similar to the Spemann organizer [63].

Serrate/Jagged are ligands for the receptor Notch. The pathway is conserved across many metazoans [64] and serves several purposes. In *Drosophila* for instance they have been linked to specifying AP identity [64], whilst loss of Notch signalling in mice causes aberrations in AP identity [65]. Strawberry notch is a downstream target within the Notch pathway. It has been shown to upregulate the pathway, pattern along the dorso-ventral wing disc and upregulate RNA pol II in the fruit fly [66].

In the fruit fly, establishment of AP axis in the oocyte requires formation of the microtubuli and actin cytoskeleton. The fruit fly tumor suppressor lethal(2) giant larvae has been shown to be required for this formation and thus required for the AP axis formation [67]. It has also been implicated in specifying posterior follicle cells in the *Drosophila* syncytium and has an impact on the proper localization of maternal morphogens Stau and Oskar. [68].

In *Drosophila* embryo development, the maternal gurken mRNA and protein is localized in the posterior cortex, while later localizing in the dorsal-anterior [69], patterning in part both the AP and DV axes [70].

The maternally provided pumilio [71] represses Hunchback posteriorly and accumulates in the pole cells, the germline forebears in *Drosophila* [71]. The germline often develops somewhat differently than somatic cells in the early embryo, and are usually specified early in development. Similarities in pumilio expression and function are found across the metazoa in *Drosophila, Xenopus, C. elegans* etc [72].

Bruno, an RNA-binding protein represses the posterior- and germline specifying Oskar in the fruit fly [73]. Oskar is a key determinant in *Drosophila* AP specification, and thus Bruno is essential as it represses Oskar throughout the embryo with the exception of the posterior. The protein also binds to the 3´ of the Gurken mRNA, repressing its localization in a similar fashion to Oskar [73]. Maternal bruno can be localized to the vegetal pole in *Xenopus* [74]

1.3 - Tardigrades as an emerging model system:

The superclade Ecdysozoa contains two of the most well-studied and well-known model organisms *Caenorhabditis elegans* and *Drosophila melanogaster* which have been ground-breaking in virtually every field of biology. The biological information on these animals and the techniques developed on them have increased our understanding of biology as a whole exponentially. Nonetheless, the understanding of related taxa such as Loricifera, Priapulida, Nematomorpha, Onychophora and Tardigrada is exceedingly small. As such, Gabriel et al. [75] postulated that tardigrades could serve as a new model system for exploring how various developmental mechanisms could lead to diverse morphologies, as the techniques and information from *C. elegans* and *D. melanogaster* could be applied to other Ecdysozoan relatives.

Tardigrades, most popularly known for being microscopic cosmopolitans with extreme survival abilities, are largely overlooked in research. Most of the studies concern their impressive, though not omnipresent, affinity for surviving extreme conditions by way of cryptobiosis [76], [77], the proposed evidence for a massive horizontal-gene-transfer [78], which was quickly refuted [79] and their phylogenetic position within the Ecdysozoa which is still debated.

In order to establish the tardigrade as a candidate model-system, questions about the genome, cell lineages and culturing methods were asked. These questions were solved in the tardigrade species of *Hypsibius dujardini* by Gabriel et al. [75]. The species was chosen due to its stereotyped nuclear migrations, asymmetric cell divisions and cell migrations, as well as their clear embryos which allowed for easy visual interpretation of cell stages and their typical rate of protein-coding sequence evolution [75]. The species is parthenogenic, in which females lay eggs that undergo meiosis and achieve diploidy by chromosome duplication rather than fertilization [80]. They have a rapid life-cycle which is suitable for culturing and lab-work [75]. In addition to these findings, several genomic and transcriptomic studies have been performed on the species, which eases further work on it [81]–[83]. All in

all, *Hypsibius dujardini* was deemed a good candidate for a suitable model species, and was thus chosen as one of the animals for this study.

1.4 - The Tardigrada within the Ecdysozoa:

Historically, the Tardigrada was grouped in the Articulata which was established by Cuvier in the work "*The Animal Kingdom"* [84]. The Articulata was formed upon the hypothesis that animals with segmented bodies were closely related, this included taxa known today as Annelida, Mollusca and Panarthropoda. In 1997 a landmark paper proposed the superclade Ecdysozoa [85], characterized by animals that molt out of their cuticle in a process called ecdysis. This clade would group together Nematoida (Nematoda and Nematomorpha), Tardigrada, Arthropoda, Onychophora, Priapulida and Kinorhyncha, with a later addition of Rotifera. Prior to this proposal the Tardigrada had been considered close relatives to Arthropoda or as members of the Aschelminthes, a now obsolete clade. The grouping with the Aschelminthes was mainly due to their similarities with Nematoida such as the triradiate pharynx, buccal stylets and their capability of cryptobiosis, specifically anhydrobiosis [86]. It is in hindsight clear that both Nematoda and Arthoropoda are members of the Ecdysozoa, and more closely related than previously thought [85], [87]–[90].

Nevertheless, the position of Tardigrada within the Ecdysozoa is not clear-cut. Numerous papers [91]–[94] have challenged the tardigrade-arthropod consensus by way of large-scale sequence alignments, and others by comparative genomics and transcriptomics [95]. Whole genome molecular phylogenies supported tardigrade-nematoid relations [83].

By textbook definition, all Panarthropods share three characters which are considered synapomorphies, which are traits shared between taxa derived through evolution of a common ancestor. This is however not consistent within the group, as one character usually is missing either in Tardigrada or Onychophora. They all have paired ventro-lateral segmental appendages, usually terminating in claws. These appendages differ between the phyla however, and the arthropods are the only ones to have undergone a true arthropodization process. Onychophorans and Arthropods also share similar gap-gene expression among the proximo-distal axis of the appendages [96], [97], this has not been observed in tardigrades. Additionally, panarthropods share a ganglionar supraesophageal brain. However, the one of tardigrades is composed of one segment, the Onychophorans of two and the Arthropods of three segments. The third character is the segmented mesoderm and mixocoel which are present in arthropods and onychophora, but not in tardigrades [98].

Another debate within the phylogenetics of the Ecdysozoa is who the Tardigrada are the sister group to. Three major hypotheses currently exist, in which the tardigrades are the sister group to either the arthropods (Tactopoda hypothesis), the arthropods and onychophorans (Lobopodia hypothesis) or to the onychophorans. Most papers will support the first two hypotheses, especially the Lobopodia hypothesis [98]–[101], as the third one has little support. Nonetheless, the general consensus points to a tardigrade-panarthropoda relationship, in which the internal phylogenies are still somewhat unresolved.

1.5 - *Terebratalia transversa* and *Pontonema vulgare*

Terebratalia transversa:

The species of *Terebratalia transversa* are members of the Brachiopoda (Lophotrochozoa, Spiralia, Protostomia). Papers have cited the members of the Lophotrochozoa as a whole as possible model systems, but have failed to consider the brachiopods [102]. Just as the tardigrades, they are far less understood and researched than the average model system, though recent strides have been made [103]–[105]. This is due in part to difficulties in keeping them in culture, which to date has not been successful, as they are aquatic filter-feeding animals. Nonetheless, they have proven useful in our lab, and have been chosen for this study to perhaps shed some light into the early embryological development of brachiopods.

Pontonema vulgare:

Pontonema vulgare are a species of free-living marine nematode. In stark contrast to their relatives *Caenorhabditis elegans* they are largely under-researched. As a potential model system they are valuable as most research within the Nemotoida are centered on *C. elegans* the *Pontonema* could provide another angle at which to look at early development and the MZT. The latter of which practically no research exists to this date. The species was chosen for this study as they are closely related to *C. elegans*, a well established model system, but are dissimilar in habitat and in early embryonic development [106] as well as providing comparison to its other Ecdysozoan relatives including the tardigrade *Hypsibius dujardini*.

2 - Materials and methods

2.1 - Culture

H. dujardini were bought from Carolina Biological (cat#133960). Cultures were maintained in Chalkley's media (NaCl 0.1 g/L, $CaCl_2$ 0.006 g/L, KCl 0.004 g/L in ddH_2O, autoclaved), media was changed every 7 days. Cultures were fed the algae *Chlorococcum sp.* coincidentally with the change of media. Tardigrade cultures were kept at 22°C (12 hrs light/ 12 hrs dark cycle) whilst the algae cultures were kept at 16°C in Jaworski's media.

Pontonema vulgare were collected at the White Sea Biological station by collaborators of the group. The nematodes were kept in Sali 2G media at 18°C and fed fish blood. *Terebratalia transversa* were collected near Seattle, Washington.

2.2 - Transcriptional inhibitor treatment:

To get a notion of when the zygotic transcripts are required for further development, tardigrade embryos were treated with the transcriptional inhibitor alpha-amanitin. Firstly, the embryos were sonicated (4 pulses of 30% amplitude for 5 seconds, 15 seconds rest) as previous runs of experimental treatment and subsequent DAPI or Hoechst staining showed that the eggs were quite impenetrable to staining. The embryos were then allowed to rest for 15 minutes on ice. The embryos were then split and treated in a gradient of 10-, 20- and 50 µg/mL alpha-amanitin in Chalkley's media for 24 hours. The embryos were then fixed in 4% paraformaldehyde (PFA) in Chalkley's for an hour at room-temperature (RT), then washed five times with PBS (18.6 mM NaH_2PO_4, 84.1 mM Na_2HPO_4, 1750 mM NaCl, pH adjusted to 7.4, autoclaved) to remove remnants of fixative solution. The samples were then treated with DAPI (cat#D1306, Invitrogen, (1 µg/mL in Chalkley's) 10 minutes then imaged by Axioscope 5/7/Vario (Zeiss).

The same procedure was applied to embryos of the nematode *Pontonema vulgare*. As the embryos of the nematode develop at a slower rate, the embryos would be treated for up to a week, with a change of their media Sali 2G with alpha-amanitin every two days. Again, the embryos were treated in a gradient of 10-, 20- and 50 µg/mL alpha-amanitin. The embryos were not sonicated, but the eggshells were penetrated prior to treatment, in order to permeabilize them. They were not treated with DAPI post-treatment as the embryos of the *Pontonema* clearly show the given cell-stage in early development.

2.3 - Construction of gene trees

To verify that the genes of interest chosen were in fact the ones they appeared to be, gene trees were constructed. Firstly, several homologs of each gene were found by BLASTing the target sequence of the *Hypsibius dujardini* genes. Candidate homologs were sampled with an emphasis on Ecdysozoan relatives and other known model-species such as mice and zebrafish. Target genes and homologs were manually analyzed by comparison of domains. The target gene, and its homologs were then arranged in a multiple-sequence alignment and the tree created by the online tool from EMBL-EBI using the maximum likelihood method ClustalW which prioritizes alignment of the most similar sequences onward to the least similar sequences to achieve global alignment [107].

2.4 - RNA isolation, cDNA synthesis, amplification and purification:

2.4.1 - Embryo sampling:

Eggs were sampled procedurally from approximately 0 hours-post-laying (hpl) to 8 hpl, with one hour intervals. Some samples contained embryos collected at random hpl solely for the purpose of synthesizing cDNA and subsequent procedures. Samples were snap-frozen in liquid nitrogen, then stored at -80°C.

2.4.2 - RNA isolation

To isolate total RNA from embryonic samples, the NucleoSpin RNA XS kit from Macherey-Nagel was used (cat#740902.50) Samples had their membranes disrupted by three consequential snap-freeze-to-heat (42°C) cycles. Lysation was achieved by drilling the samples, approximately 150 embryos per treatment, in 200 µL buffer RA1 supplemented with 4 µL TCEP . The lysate is then transferred unto a NucleoSpin Filter in a 2 mL collection tube then centrifuged (30s, 11.000 rpm). Following discardment of the NucleoSpin Filter, 200 µL 70% ethanol was added to the lysate and subsequent transfer of the lysate to a NucleoSpin RNA XS Filter in a collection tube, centrifuged (30s, 11.000 rpm). The collection tube was replaced followed by addition of 100 µL buffer MDB to the column, then centrifuged (30s, 11.000 rpm).

To the column, 25 µL of rDNase reaction mixture (of which 3 µL rDNase and 25 µL rDNase Reaction Buffer, mixed) was added and incubated for 15 min at RT. Following this was a series of washes, 100 µL RA2 incubated for 2 min at RT then centrifuged (30s, 11.000 rpm), 200 µL RA3 then centrifugation (30s, 11.000 rpm), 200 µL RA3 then centrifugation (2 min,

11.000 rpm). The column was then transferred to a nuclease-free 1.5 mL tube. RNA was then eluted by addition of 10 µL nuclease-free H_2O and centrifugation (30s, 11.000 rpm). The concentration was measured by NanoDrop.

2.4.3 - cDNA synthesis:

To synthesize cDNA from the isolated embryonic RNA, the SuperScript III First-Strand Synthesis System for RT-PCR kit was used (cat#18080-051, Invitrogen). For each reaction, 8 µL RNA, 5 ng/µL random hexamers and 1 mM dNTPs in 10 µL H_2O were incubated at 65°C for 5 minutes, then placed on ice for 1 minute. To each reaction a mixture containing 2 µL 10X RT Buffer , 10 mM $MgCl_2$, 20 mM DTT, 40 U RNaseOUT , 200 U SuperScript III RT to 10 µL was added. The mixture was incubated at 25°C for 10 minutes followed by 50°C for 50 minutes, the reactions were terminated at 85°C for 5 minutes, all incubations performed in an Eppendorf Thermocycler. To each reaction 1 µL of RNAse H (cat#AM2293, Invitrogen) was added and incubated at 37°C for 20 min. The concentration was measured by NanoDrop and stored at -20°C.

2.4.4 - Amplification PCR:

To amplify and purify the DNA of interest, the cDNA was used in a PCR with primers designed in Macvector version 18.1.3 and ordered from Sigma Aldrich, sequences can be found in the Appendix. Each reaction was composed and run in an Eppendorf Thermocycler according to Table 2.1.

Table 2.1: Amplification PCR

AMPLIFICATION PCR		PCR PROGRAM		
Content	**[C] / volume**	**Step**	**T (°C)**	**Time**
5x Buffer	5 µL	1	94	5 min
$MgCl_2$	7.5 mM	2	94	30 s
dNTP	500 µM	3	65	30 s
Taq polymerase	0.15 µL/0.75 u	4	72	2 min
Template cDNA	1 µL	5	REPEAT 2-4 34x	
Primer Forward	1.5 µM	6	72	10 min
Primer Reverse	1.5 µM	7	4	-
Total	**25 µL**			

Each reaction was verified to match accordingly to their expected length upon a 1% agarose gel containing SybrSafe (cat#SS33102, Thermo Fisher) as well as 5 µL GeneRuler 1kb Plus DNA Ladder (cat#SM1333, Thermo Fisher) for 45 minutes at 90V.

Reagents that have no listed concentration are listed in Table 2.2 in further detail.

Table 2.2: PCR reagents

PCR REAGENTS		
Product	**Supplier**	**#Cat number**
5X Buffer	Promega	M8911
Taq Polymerase	Promega	M7805
BigDye v1.1	ThermoFisher	4337452
5X Sequencing buffer	ThermoFisher	4336697

2.4.5 - PCR purification:

The PCR product was then purified by way of Qiagen's MiniElute PCR Purification Kit (cat#28004, Qiagen). To one volume of PCR product, five volumes of Buffer PB were added and mixed. The solutions were transferred to a MiniElute Column in a collection tube and centrifuged for 1 minute. The flow-through was discarded, and 750 µL Buffer PE was added to the column, centrifuged for 1 minute. The column was transferred onto a new 1.5 mL tube. To elute the DNA, 10 µL Buffer EB was added to the column, incubated for 1 minute at RT then centrifuged. The eluted DNA was measured by NanoDrop.

2.5 - Ligation and heat-shock transformation

2.5.1 - Ligation

Each purified PCR product (1,5 µL) was ligated in 3,5 µL 2X Ligation Buffer (cat#A3600, Promega), 0,5 µL pGEM-T Easy Vector (cat#A3600, Promega) and 0,5 µL T4 DNA Ligase (cat#A3600, Promega) for an hour a RT.

2.5.2 - Heat-shock transformation and plating

Following ligation reaction 2 µL of the ligation mix was carefully mixed with thawed 50 µL *E. coli* cells in prechilled 1.5 mL tubes and incubated on ice for 20 minutes. The ligation/*E. coli* mix was heat-shocked at 42°C for 50 seconds then transferred back to ice for 2 minutes. Following this was an addition of 450 µL prevarmed ampicillin-LB media, incubated at 37°C

whilst shaking (450 rpm, Eppendorf Thermocycler) for an hour. 200 µL of the bacteria/ligation mix were plated upon X-gal inoculated ampicillin-LB plates at 37°C overnight. LB-plates are made accordingly; to 0.5 L H_2O, 12.5 g LB and 7.5 g agar were added, mixed and autoclaved. The solution was then kept at 60°C followed by addition of 500 µL ampicillin, mixing and plating of LB-ampicillin.

2.6 - Colony PCR and Miniprep

2.6.1 - Colony PCR

Colony PCR was performed in triplicates according to Table 2.2.

Table 2.2: Colony PCR

COLONY PCR		PCR PROGRAM		
Content	**[C] / volume**	**Step**	**T (°C)**	**Time**
H_2O	15.85 µL	1	94	5 min
5x Buffer	5 µL	2	94	30 s
$MgCl_2$	3mM	3	50	30 s
dNTP	200µM	4	72	1:45 min
T7 primer	0.5µM	5	REPEAT 2-4 34x	
SP6 primer	0.5µM	6	72	10 min
Taq polymerase	0.15 µL / 0.75 u	7	4	-
Colony bit	1			
Total	**25 µL**			

Following colony PCR 5 µL of each reaction was loaded onto an 1% agarose gel containing Sybr Safe as well as 5 µL GeneRuler 1kb Plus DNA Ladder, ran at 90V for 45 min.

2.6.2 - Miniprep

The same bacteria picked for colony PCR was used to inoculate 2 mL of ampicillin-LB media (0.5 L H_2O, 12.5 g LB, autoclaved), left shaking at 210 rpm, 37°C overnight. Plasmid DNA was purified from appropriate cultures by miniprep. Firstly 1.5 mL of culture was transferred to an 1.5 mL tube and centrifuged (30s, 11.000 rpm). The supernatant was discarded followed by addition of 250 µL buffer A1 (cat#740911.1, Macherey Nagel) and resuspension of the pellet. 250 µL buffer A2 (cat#740912.3, Macherey Nagel), mixed gently and incubated

at RT, 5 min. 300 µL buffer A3 (cat#740913.1, Macherey Nagel) was added and mixed gently. The solution was centrifuged (5 min, 11.000 rpm). The supernatant was then transferred to NucleoSpin Plasmid Columns (cat#740588.50S, Macherel Nagel) followed by centrifugation (1 min, 11.000 rpm) and discardment of the flow-through. 600 µL buffer A4 (cat#740914.1, Macherey Nagel) was added to the column followed by centrifugation (1 min, 11.000 rpm) and discardment of the flow-through. Centrifugation was repeated (2 min, 11.000 rpm) and flow-through discarded. Plasmid DNA was then eluted by the addition of 50 µL buffer AE (cat#740917.1, Macherey Nagel) to the column, incubated 1 min at RT followed by centrifugation (1 min, 11.000 rpm).

2.7 - Sequencing

Each gene of interest and their corresponding miniprep product were then sequenced according to Table 1.3. Each reaction was performed in duplicates and sequenced with two different primers, T7 and SP6.

Table 2.3: Sequencing PCR

SEQUENCING PCR		PCR PROGRAM		
Content	**[C] / volume**	**Step**	**T (°C)**	**Time**
H_2O	3.8	1	95	5 min
Big Dye v3.1	1 µL	2	95	10 s
5x Seq. Buffer	1 µL	3	50	5 s
Primer T7/SP6	0.32 µM	4	60	4 min
Plasmid DNA	1 µL	5	REPEAT 2-4 24x	
Total	**10 µL**	6	4	-

Following Sanger sequencing, each gene was verified in Macvector version 18.1.3 for whichever of the two enzymes T7 or SP6 would synthesize the antisense riboprobe as described in section 2.8.

2.8 - Probe PCR and riboprobe-synthesis

2.8.1 - Probe PCR

For each probe to be synthesized a linearization reaction was prepared according to Table 1.3.

Table 2.3: Probe PCR

PROBE PCR		PCR PROGRAM		
Content	**[C] / volume**	**Step**	**T (°C)**	**Time**
H_2O	30.7 µL	1	94	5 min
5x Buffer	1x	2	94	30 s
$MgCl_2$	3mM	3	50	30 s
dNTP	200µM	4	72	1:45 min
T7 primer	1µM	5	REPEAT 2-4 39x	
SP6 primer	1µM	6	72	10 min
Taq polymerase	0.3 µL	7	4	-
MP DNA	1 µL			
Total	**50 µL**			

Following the PCR, the products were verified upon an 1% agarose gel (with SYBR Safe) and purified according to section 2.3.4.

2.8.2 - Riboprobe synthesis

Following Sanger sequencing, sequences were confirmed

To synthesize probes a reaction (7.5 mM ATP, 7.5 mM GTP, 7.5 mM CTP, 6 mM UTP, 52.5 nM Dig-UTP, 2.1 µL linearized DNA, 1 µL 10X Buffer (cat#AM1334, Invitrogen), 1 µL SP6/T7 enzyme mix (cat#AM1334, Invitrogen)) was prepared in 0.2 mL tubes and incubated at 37°C for 5 hours. Following this, 0.5 µL RNAse-free DNAse I (cat#AM1334, Invitrogen) was added to each reaction, incubated for 15 minutes at 37°C. To each reaction 10 µL RNAse-free water and 10 µL lithium chloride precipitation solution (cat#AM1334, Invitrogen) followed by incubation at -20°C for 30 minutes. The solutions were then centrifuged at 9000 rpm for 15 minutes at 4°C. The supernatant was removed and the pellet washed with 500 µL 70% RNAse-free EtOH (in DEPC H_2O). The solutions were then centrifuged once more at 4°C for 15 minutes. The supernatant was removed and the pellet was resuspended in 25 µL preheated 60°C RNAsecure (cat#AM7005, ThermoFisher), the solutions were then

incubated at 60°C for 10 minutes. The probes then had their concentration measured by NanoDrop. This is the protocol for probes used in both the brachiopod *T. transversa* and the tardigrade *H. dujardini*, however the probes for the brachiopod were provided by former and current group members.

2.9 - In Situ Hybridization

2.9.1 - *Terebratalia transversa* in-situ

Pre-fixed samples of *Terebratalia transversa* (fixed in 4% paraformaldehyde) already in MeOH were rehydrated through one wash of 60% MeOH/40% PTw (1X PBS, 0.1% Tween-20, DEPC treated) and another wash of 30% MeOH/70% PTw followed by four washes of PTw. The samples were then treated with Proteinase K (0.01 mg/mL in PTw, 10 minutes). The digestion was then stopped by two washes of glycine (2 mg/mL in PTw). Samples were then washed once in 1% TAE (triethanolamine (in PTw)) followed by another wash of 1% TAE containing 0.3% acetic anhydride, followed by an addition of acetic anhydride to a concentration of 0.6% per well. This was followed by two washes of PTw and a refixation in formaldehyde (4% in PTw) for an hour. This was followed by five washes of PTw, in which the last wash was set to 80°C for 10 minutes. Samples were washed with preheated 67°C hybe buffer (50% formadide, 5X SSC pH 4.5, 50 µg/mL heparin, 0.1% Tween-20, 100 µg/mL salmon sperm DNA and DEPC treated H_2O to final volume 40mL) for 10 minutes. The hybe buffer was replaced and the samples were incubated overnight at 67°C. The following day the riboprobes (1 ng/µL in hybe buffer) were denatured for 10 minutes at 80°C. The hybe buffer was replaced with riboprobes and left to hybridize at 67°C for 48 hours.

Embryos had their riboprobe/hybe solution removed and replaced with hybe wash (50% formadide, 5X SSC pH 4.5, 0.1% Tween-20 and DEPC treated H_2O to final volume 40mL), washed at 67°C for 10 minutes followed by another wash in hybe wash for 40 minutes at 67°C. This was followed by a wash of 75% hybe wash/25% 2X SSC (cat#S6639, Sigma-Aldrich, pH 7.0), a wash of 50% hybe wash/50% 2X SSC, a wash of 25% hybe wash/75% 2X SSC and a wash of 100% 2X SSC. All washes were at 67°C for 30 minutes. Following this was a set of three 20 minute washes of 0.2X SSC at 67°C and a series of washes, all performed for 10 minutes at room temperature, of 75% 0.2X SSC/25% PTw, 50% 0.2X SSC/50% PTw, 25% 0.2X SSC/75% PTw and a final wash in 100% PTw.

To visualize the probe the samples were washed five times in PBT (1X PBS, 0.2% Triton X-100, 0.1% BSA) at room temperature then blocked in Boehringer-Mannheim blocking

buffer in maleic acid buffer (MAB: 100 mM maleic acid, 150 mM NaCl, pH 7.5) for an hour at room temperature and incubated overnight at 4°C in Boehringer-Mannheim blocking buffer (in maleic acid buffer + Anti-Dig (1:5000)). The samples were then washed five times in PBT for 15 minutes and five times in PTw for 30 minutes and left to develop overnight at 4°C.

Samples were washed three times in AP buffer without $MgCl_2$ (100 mM NaCl, 100 mM Tris (pH 9.5), 0.5% Tween-20 in DEPC H_2O) for 10 minutes. Samples were washed in AP buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris (pH 9.5), 0.5% Tween-20 in DEPC H_2O) for 10 minutes. The signal was then developed in fresh AP substrate solution (AP buffer + 0.165 mg/mL NBT (cat#11383213001, Roche), 0.165 mg/mL BCIP (cat#1681460001, Roche) at room temperature. The AP substrate solution was exchanged twice a day awaiting development of the signal.

Once the signal had appeared, the reaction was stopped by 2 washes of AP buffer without $MgCl_2$ followed by 5 washes of PTw. To remove background staining the samples were washed 3 times with 100% EtOH followed by another wash of 100% for 15 minutes. The samples were then washed for 10 minutes each in 75% EtOH/25% PTw, 50% EtOH/50% PTw, 25% EtOH/75% PTw followed by 3 10 minutes washes of PTw. The PTw was then exchanged for 70% glycerol (in PTw) containing 1 µg/mL DAPI. Samples were mounted and imaged by Axioscope (Zeiss).

2.9.2 - Tardigrade in-situ

A different protocol for in situ in the tardigrade was attempted as the standard protocol described above for *Terebratalia transversa* did not yield any results.

The in situ method for the tardigrade *Hypsibius dujardini* was posed upon the protocol for the closely related *Hypsibius exemplaris* [108], [109]. Embryos were transferred to 1 mL of 0.5X PTw and centrifuged for 3 minutes at 11.000 rpm. As much of the 0.5X PTw was removed, followed by addition of 20 µL chitinase/chymotrypsin permeabilization solution (5 U/mL chitinase, 10 mg/mL chymotrypsin in 0.5X PTw), incubated for an hour at RT. The embryo/permeabilization solution was centrifuged for 3 minutes at 11.000 rpm. The embryos were washed three times as such; addition of 500 µL 0.5X PTw, incubated for 5 minutes followed by centrifugation at 11.000 rpm for 3 minutes and removal of superfluous liquid. This was followed by fixation with 1 mL of fixative solution (4% formaldehyde, 0.1% Tween-20, 33% heptane to 1 mL in 0.5X PTw), rocking at 500 rpm for 30 minutes. The fixed embryos were then centrifuged once at 11.000 rpm for 3 minutes and washed five times, just

as the previous washes in 500 µL of 0.5X PTw.

Samples were then transferred to a 0.5X PTw pre-washed Mobicol column (catM1050, MoBiTec) enclosed with a Luer-lock cap with a 1 mL syringe. The embryos were washed for a series of washes of 5 minutes in 25% methanol in 0.5X PTw, 50% methanol in 0.5X PTw, 75% methanol in PTw, 90% methanol in PTw and 100% methanol followed by three washes of 100% methanol. The samples were stored at -20°C for a minimum of 20 minutes followed by washes in a series of five minute washes of 90% methanol in 0.5X PTw, 70% methanol in 0.5X PTw, 50% methanol in 0.5X PTw, 25% methanol in 0.5X PTw and three washes of 0.5X PTw.

Embryos were transferred from to a dish and cut out of their eggshells by a 0.25G needle, transferred to a fresh Mobicol column and washed with 0.5 X PTw. Samples were washed in 1% TAE (in 0.5X PTw) for 5 minutes and washed twice for 5 minutes in 1% TAE (containing 0.7% acetic anhydride, in 0.5X PTw) and thrice with 0.5X PTw. The embryos were then washed twice for 20 minutes with hybe buffer at RT followed by another wash with hyb buffer preheated to 60°C at 60°C for 2 hours.

DIG-labeled probes were diluted to 1 ng/µL in hybe buffer, denatured at 100°C for 5 minutes. The probes were added to the columns, and left to hybridize overnight at 60°C. The samples were washed five times quickly and five times for 5 minutes with hybe wash. Embryos were then washed five times quickly and once for 30 minutes with 2X SSC + 0.1% Tween-20. The embryos were washed twice for 30 minutes with 1X SSC + 0.1% Tween-20 followed by two washes of 30 minutes with 0.2X SSC + 0.1% Tween-20. All washes done at 60°C.

The embryos were then washed with RT 0.5X PTw followed by incubation for two hours at RT in Boehringer-Mannheim blocking buffer in MAB. This was followed by incubation overnight at 4°C in Boehringer-Mannheim (in MAB, containing anti-DIG antibody, 1:1500). Embryos were then washed five times quickly and five times for 10 minutes with MAB and three times quickly and once for 10 minutes in AP buffer followed by transferral to a well plate. The embryos were developed in AP substrate solution. Reaction was stopped by two washes of AP buffer without $MgCl_2$ followed by five washes of PTw.

3 - Results

3.1 - Alpha-amanitin treatment in *P. vulgare* and *H. dujardini* arrest the cell cycle after the fourth and fifth cleavage

Figure 3.1 shows the results of the transcriptional inhibition experiments in the nematode *Pontonema vulgare*.

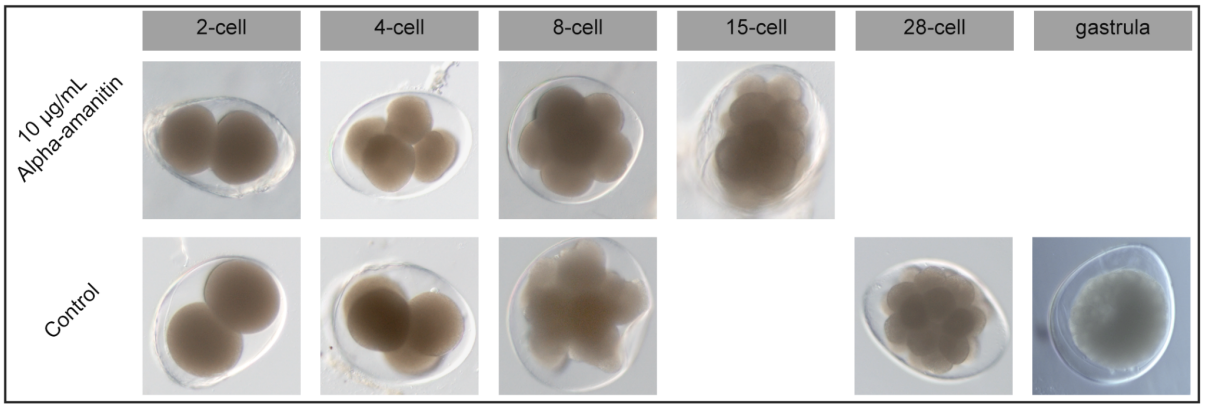

Figure 3.1.1 - Transcriptional inhibition in the nematode *Pontonema vulgare*
The top row shows live embryos in presence of the transcriptional inhibitor alpha-amanitin. The bottom row shows the control. In the presence of 10 µg/mL alpha-amanitin, cell cycle cleavages were arrested after the 15-cell stage. The control continued dividing up until the gastrula stage.

Prior to this series of experiments, a gradient of 10-, 20- and 50 µg/mL alpha-amanitin was attempted on *Pontonema vulgare* embryos; these are not shown. These experiments all showed the same results, and as such a concentration of 10 µg/mL of alpha-amanitin was chosen for this procedure. Embryos that were transcriptionally inhibited stopped dividing following the 15-cell stage, or after the fourth cleavage. One embryo, out of 60, however developed until the 28-cell stage (not shown), this embryo was however severely deformed. The control developed until the gastrula stage and onward (not shown).

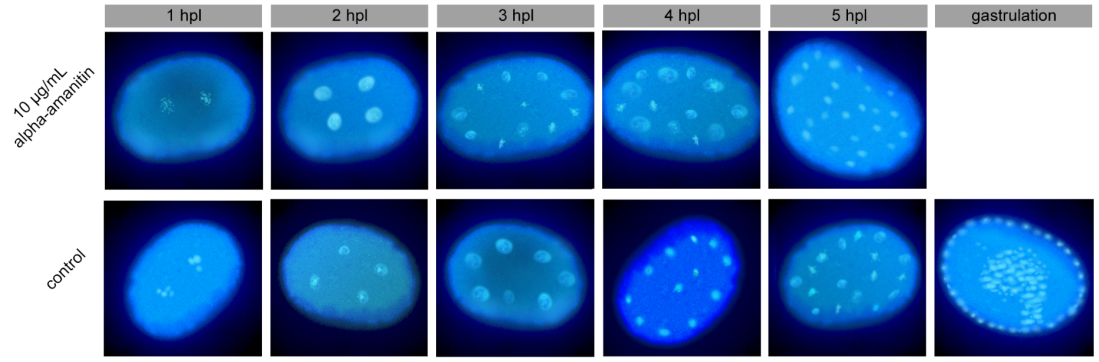

Figure 3.1.2 - Transcriptional inhibition in the tardigrade *Hypsibius dujardini*

Embryos live stained with 1 µg/mL DAPI. Embryos were sonicated, 4 pulses of 30% amplitude for 5 seconds and 15 seconds rest intervals, prior to staining. The top row shows the experimental treatment with 10 µg/mL alpha-amanitin and the bottom row shows the control. Embryos were sampled each hour-post laying (hpl), as the embryos typically cleave every hour or so.

Prior to this series of experiments, a gradient of 10-, 20- and 50 µg/mL alpha-amanitin was attempted on *Hypsibius dujardini* embryos; these are not shown. These experiments all showed the same results, and as such a concentration of 10 µg/mL of alpha-amanitin was chosen for this procedure. Embryos that were exposed to the transcriptional inhibitor alpha-amanitin had their cell cycles arrested following the fifth cleavage cycle. The control developed further until the gastrula and onward (not shown). The single cell stage, or 0 hpl is not observed as the procedures of sonication and DAPI staining do not allow for sufficient time for capturing this stage.

3.2 - Gene trees for *Hypsibius dujardini* target genes show mostly ingrouping with homologs

The gene trees for tardigrade target genes constructed as described in section 2.3 are shown in figure 3.2

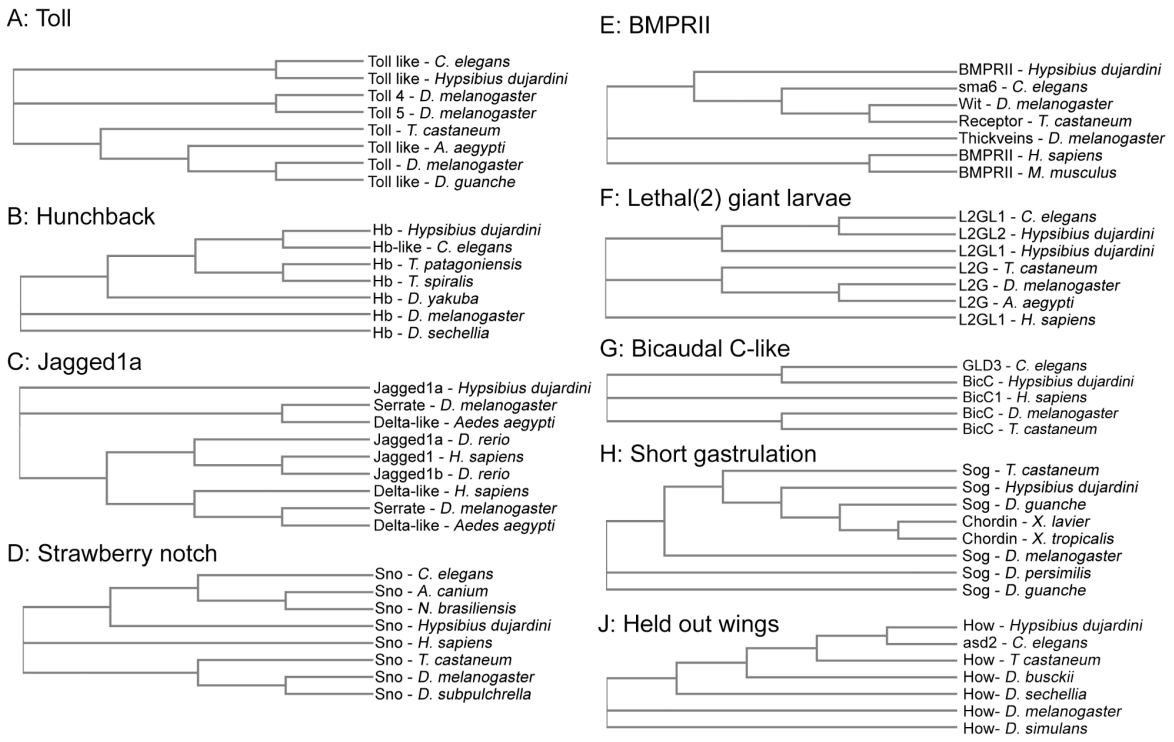

Figure 3.2 - Gene trees for candidate in-situ tardigrade genes and homologs

The tree shows the target genes toll, hunchback, Jagged1a, strawberry notch, BMPRII, lethal(2) giant larvae, bicaudal C-like, short gastrulation, held out wings and a select few homologs of each gene. The homologs are derived from *Caenohabditis elegans, Drosophila melanogaster, Drosophila guanche, Drosophila subpulchrella, Drosophila simulans, Drosophila sechellia, Drosophila busckii, Drosophila persimilis, Drosophila yakuba, Aedes aegypti, Tribolium castaneum, Trichinella patagoniensis, Trichinella spiralis, Nippostrongylus brasiliensis*, *Ancylostoma caninum, Xenopus laevis, Xenopus tropicalis, Mus musculus* and *Homo sapiens.*

Most target genes shown in A-J seem to group neatly with its counterparts, with the exception of the tardigrade Jagged1a as shown in C. For the target gene Frizzled7 (Frz7) no homologs with viable confidence were found other than its seemingly close relative Frizzled in *Drosophila melanogaster*.

For toll-like it is paired most closely to the toll-like of *C. elegans* whilst the toll receptors in arthropods group mostly together within themselves. The *H. dujardini* hunchback gene is nestled again with the homolog from *C. elegans*. The two hb homologs of *Trichinella*, species of parasitic roundworms, pair together. Hb in *D. yakuba, D. sechellia* and *D.*

melanogaster are all somewhat separate from the other homologs.

The *H. dujardini* jagged1a was found to be homologous with variants of other jagged, delta and serrate genes. Interestingly the tardigrade jagged1a is out grouped from its counterparts. In the case of strawberry notch the tardigrade variant is nestled closely to nematode variants from *C. elegans, A. caninum* and *N. brasiliensis*. The human variant is separate whilst the arthropod variants are grouped together.

H. dujardini BMPRII is nestled with sma6 (*C. elegans*), wit (*D. melanogaster*) and an unannotated receptor from *T. castaneum*. All are variants of the TGF-beta receptor subfamily. BMPRII from both *H. sapiens* and *M. musculus* group together, whilst the *D. melanogaster* derived thickveins groups separately. Lethal(2) giant larvae-like 1 and 2 from *H. dujardini* group with its *C. elegans* homolog. The arthropod variants group together, whilst the human variant is out grouped. Bicaudal C-like from *H. dujardini* groups with the *C. elegans* GLD-3, a bicaudal C homolog. The two arthropod variants group together, whilst the human variant out groups.

Tardigrade sog groups with the variants from *T. castaneum* and *D. guanche* and the chordins from *X. lavier* and *X. tropicalis*. The three variants from *D. melanogaster, D. sechellia* and *D. simulans* are all separate in this tree. How is nestled closely with asd-2 (*C. elegans*), less close to how variants from *T. castaneum, D. busckii* and *D. sechellia*. The *D. melanogaster* and *D. simulans* how variants outgroup.

3.3 - RNA and cDNA concentrations

The results of the RNA isolation procedures were exceedingly low and of relatively poor quality. Approximately 150 embryos were used for each extraction by way of the Macherey-Nagel RNA XS kit. Nevertheless, the RNA proved sufficient for downstream purposes such as cDNA synthesis, as shown in the high concentrations and good quality.

Upon eggshell penetration and RNA isolation of tardigrade embryos, the concentrations were measured by NanoDrop to be 2.6 ng/µL in the first attempt, 2.3 ng/µL after the second attempt and 5.4 ng/µL in the third attempt. Following cDNA synthesis the concentration was measured by NanoDrop and found to be 582 ng/µL in the first attempt, 782 ng/µL after the second and 1059 ng/µL after the third attempt.

3.4 - Amplification PCR:

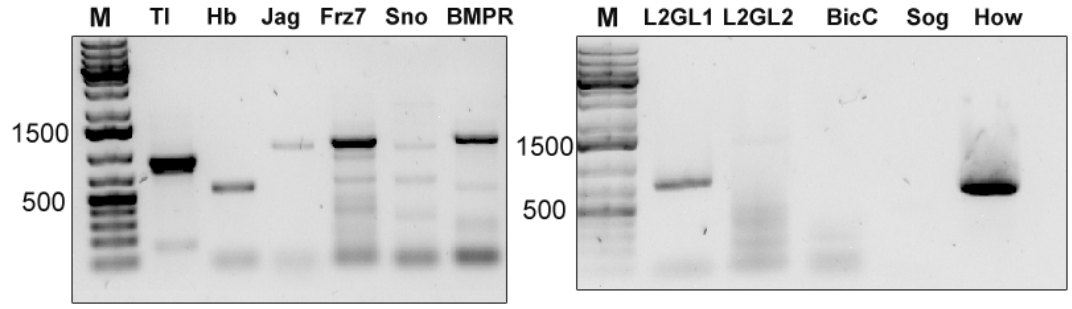

Figure 3.4.1 - Amplification PCR and gel-analysis of cDNA

Pictured above are 1% agarose gels stained with SYBR Safe. The left-most bands are GeneRuler 1kb Plus DNA ladders (ThermoFisher) with the 1500 and 500 bp bands marked. cDNA was amplified by primers specific to genes of interest, from left to right: Toll, Hunchback, Jagged-1A, Frizzled7, Strawberry notch, BMPRII, lethal-2-giant larvae like 1, lethal-2-giant larvae like 2, Bicaudal C-like, short gastrulation and held out wings. Primers can be found in the appendix.

Bands that were consistent with expected lengths and of sufficient intensity were purified. This includes Tl, Hb, Jag, Frz7, BMPR and How. Sno, L2GL1, L2GL2, BicC and Sog showed poor results in regard to expected length and intensity on the gel. The expected lengths of the successful reactions are Tl: 804, Hb: 575, Jag: 1113, Frz7: 957, BMPR: 1215, How: 683.

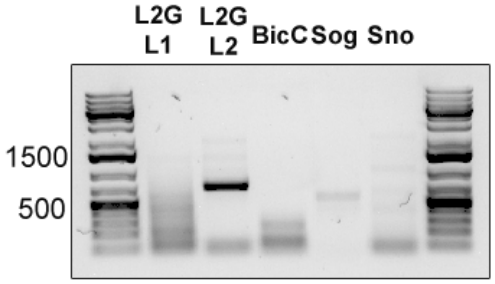

Figure 3.4.2 - Amplification PCR and gel-analysis of cDNA

Pictured above are 1% agarose gels stained with SYBR Safe. The left-most bands are GeneRuler 1kb Plus DNA ladders (ThermoFisher) with the 1500 and 500 bp bands marked. cDNA was amplified by primers specific to genes of interest, from left to right: lethal-2-giant larvae like 1, lethal-2-giant larvae like 2, Bicaudal C-like, short gastrulation and strawberry notch. Primers can be found in the appendix.

In an attempt to extract more genes of interest, a new batch of cDNA was used for this PCR and gel-analysis. However only L2GL2 was deemed of sufficient quality as it matched its expected length of 753 bp.

3.5 - Bacterial transformation and colony PCR:

Transformation of bacterial cultures with plasmid ligated genes of interest all proved successful, with the exception of Frizzled7. Colonies from these cultures were then used in colony PCR and gel-electrophoresis as shown in Figure 3.4. Frizzled7 and L2GL2 were later used in transformation, then subsequently colony PCR and gel-electrophoresis as shown in Figure 3.13.

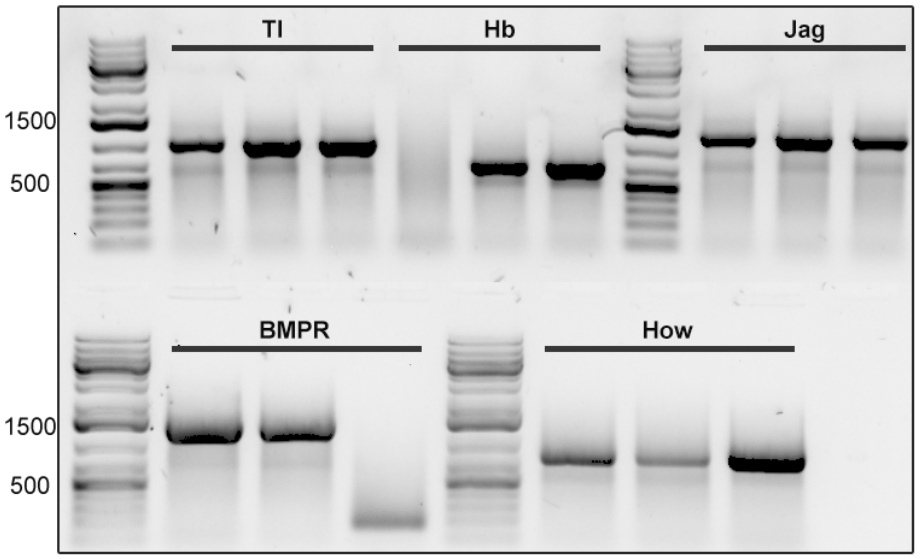

Figure 3.5.1 - Colony PCR; Tl, Hb, Jag, BMPR & How

Pictured above are 1% agarose gels stained with SYBR Safe. The left-most bands are GeneRuler 1kb Plus DNA ladders (ThermoFisher) with the 1500 and 500 bp bands marked. Following transformation of bacteria, PCR and gel electrophoresis was run to verify and confirm proper transformation of colonies. For each gene, three separate colonies were chosen. Primers used are T7 and SP6 which correspond to promoter sites on the plasmid pGEM-T Easy Vector.

PCR and gel-electrophoresis (fig. 3.5.1) showed that all colonies with the exception of the first Hunchback-transformed colony and the third BMPRII-transformed colony were successful in terms of the ligation of the gene-vector construct and the bacteria expressing said construct as they matched their expected length, Tl: 804, Hb: 575, Jag: 1113, How: 683, BMPR: 1215. With the exception of these two, ampicillin-LB media was inoculated for MiniPrep as described in 2.6.2.

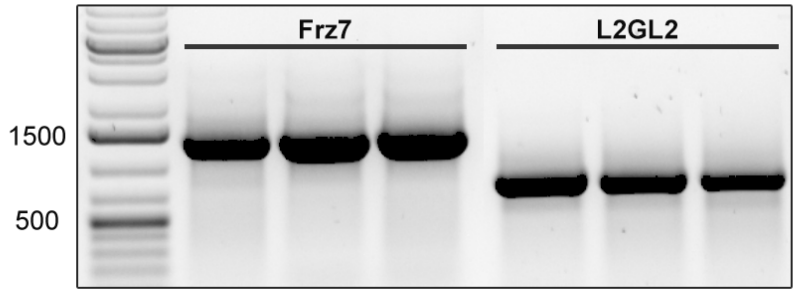

Figure 3.5.2- Colony PCR; Frz7 & L2GL2

Pictured above are 1% agarose gels stained with SYBR Safe. The left-most bands are GeneRuler 1kb Plus DNA ladders (ThermoFisher) with the 1500 and 500 bp bands marked. Following transformation of bacteria, PCR and gel electrophoresis was run to verify and confirm proper transformation of colonies. For each gene, three separate colonies were chosen. Primers used are T7 and SP6 which correspond to promoter sites on the plasmid pGEM-T Easy Vector.

PCR and gel-electrophoresis (fig. 3.5.2) showed that all colonies checked were successful in terms of the ligation of the gene-vector construct and the bacteria expressing said construct as they matched their expected length, Frz7: 957, L2GL2: 753. These colonies were then used in the inoculation of ampicillin-LB media used for MiniPrep as described in section 2.6.2.

3.6 - Probe PCR

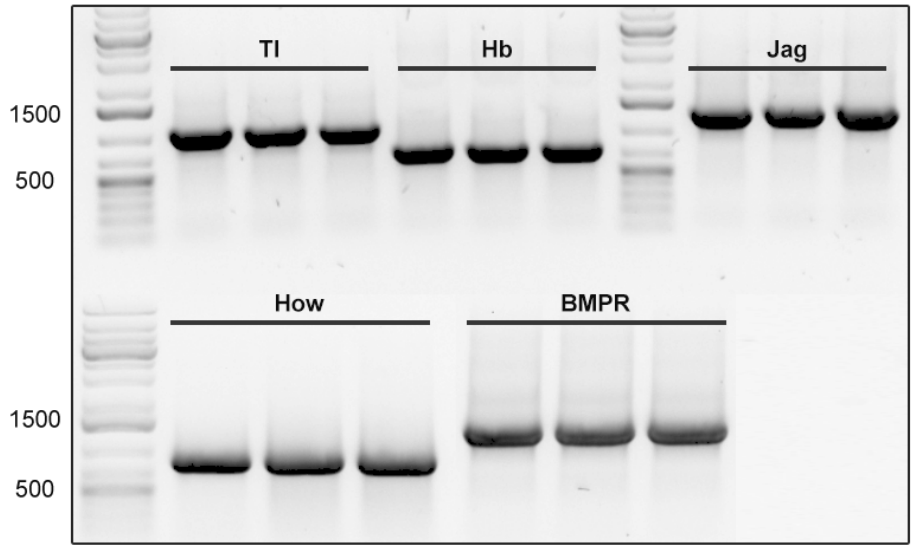

Figure 3.6.1 - Probe PCR; Tl, Hb, Jag, How & BMPR

Pictured above are 1% agarose gels stained with SYBR Safe. The left-most bands are GeneRuler 1kb Plus DNA ladders (ThermoFisher) with the 1500 and 500 bp bands marked. Primers used are T7 and SP6 which correspond to promoter sites on the plasmid pGEM-T Easy Vector.

Following the linearization reaction, or probe PCR as described in section 2.8.1, the products were checked upon an 1% agarose gel (fig. 3.6.1). All bands matched their expected lengths.Tl: 804, Hb: 575, Jag: 1113, How: 683, BMPR: 1215.

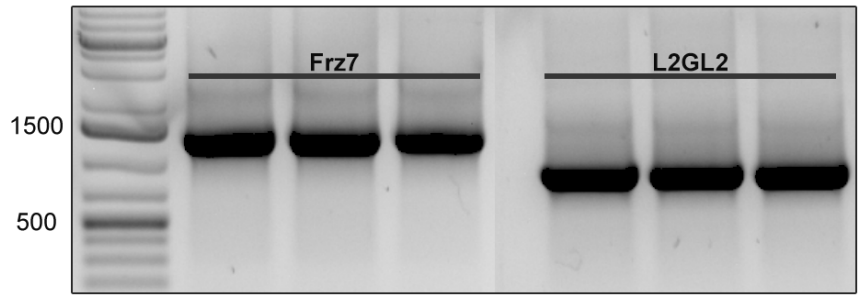

Figure 3.6.2 - Probe PCR: Frz7 & L2GL2

Pictured above are 1% agarose gels stained with SYBR Safe. The left-most bands are GeneRuler 1kb Plus DNA ladders (ThermoFisher) with the 1500 and 500 bp bands marked. Primers used are T7 and SP6 which correspond to promoter sites on the plasmid pGEM-T Easy Vector.

Following the linearization reaction, or probe PCR as described in 2.8.1, the products were checked upon an 1% agarose gel (fig. 3.6.2). All reactions matched their expected lengths, Frz7: 957, L2GL2: 753.

3.7 - Riboprobe synthesis:

Following riboprobe synthesis, the probes had their concentration measured by NanoDrop, results are shown in Table 3.1. All but the Frz7 probes showed sufficient concentrations.

Table 3.1 - Riboprobe concentration

PROBE	**[C] ng/µL**
Toll (Tl)	1339
Hunchback (Hb)	354
Jagged1a (Jag)	1405
Held out wings (How)	1114
BMPRII (BMPR)	1375
Lethal-2-giant larvae like 2 (L2GL2)	1492
Frizzled7 (Frz7)	98

3.8 - In-situ hybridization in *Hypsibius dujardini* and *Pontonema vulgare*

3.8.1 - Failed in-situ in *H. dujardini*

Both the standard and the *H. exemplaris* specific in-situ hybridization (ISH) techniques were attempted in embryos of the tardigrade *Hypsibius dujardini*. Unfortunately neither protocol yielded any results to speak of.

3.8.2 - *Terebratalia transversa* in-situ

Figure 3.8 shows the results of the ISH in *Terebratalia transversa*.

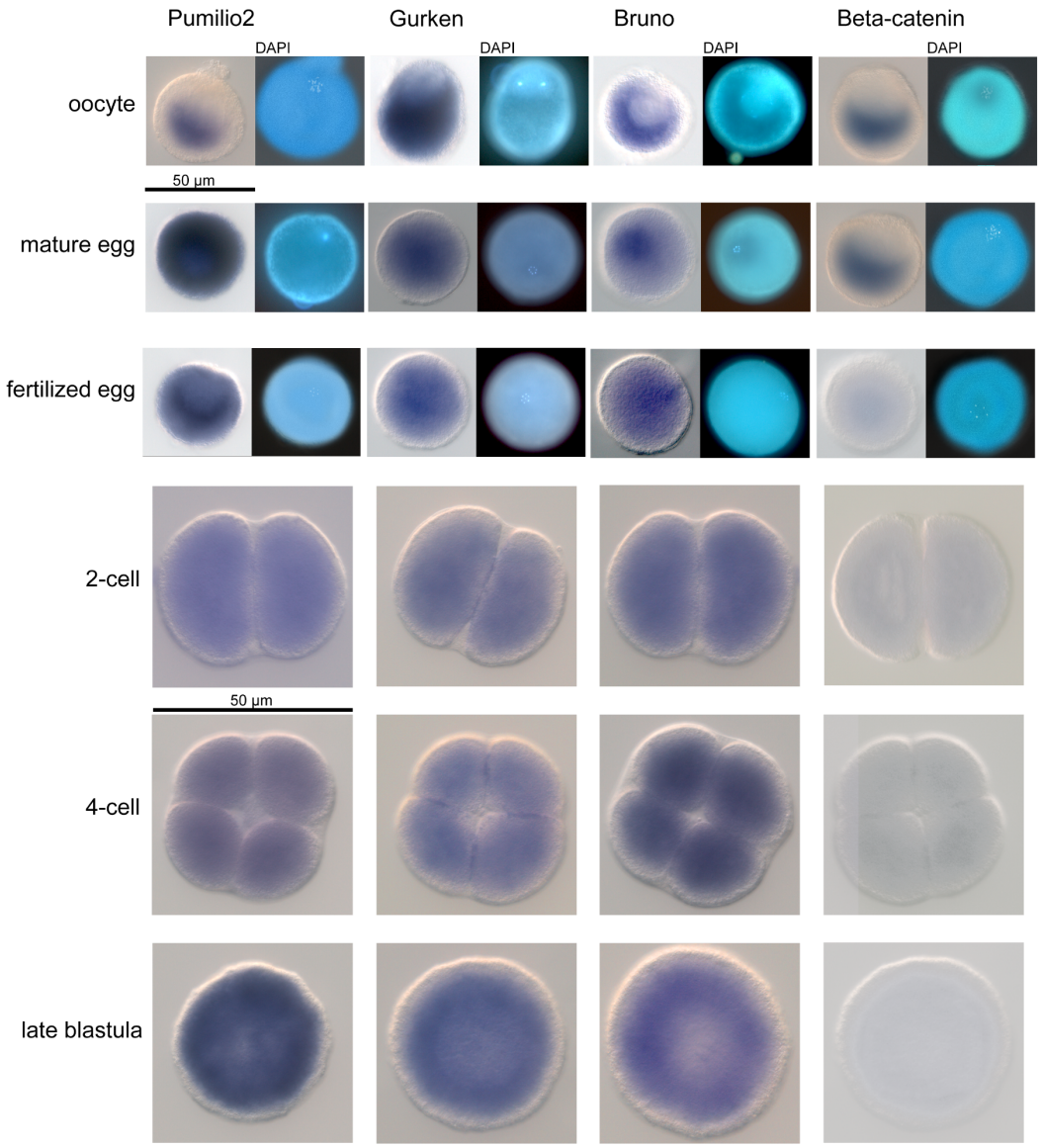

Figure 3.8 - In-situ hybridization in *Terebratalia transversa* embryos

The figure shows the results of ISH hybridization performed in the brachiopod *Terebratalia transversa*. Developmental stages are noted, from oocyte, mature egg, fertilized egg, 2-cell, 4-cell and late blastula. Embryos in the oocyte to egg stages were stained with 1 µg/mL DAPI.

Pumilio2 localized to one region, the vegetal region, in the oocyte. In the fertilized egg the signal is seen through the cell, but is expressed more strongly in an arch at the posterior. In the mature egg, the signal was overdeveloped and any specific localization was lost, expression is however throughout the cell. The signal is also observed uniformly through the 2-cell, 4-cell and late blastula stages.

Gurken is present throughout the embryos of *Terebratalia transversa* from oocyte to late blastula. In the oocyte it is present throughout the cell, but localized heavily towards the posterior pole. Expression in the remaining stages was observed to be uniform.

Bruno is expressed throughout in all stages observed. In the oocyte it is observed in the vegetal pole, while in the mature egg it appears to have relocalized to the animal pole. In the fertilized egg and onwards the expression is even throughout the cells.

Beta-catenin is expressed in the oocyte and the mature egg. In both stages the expression is localized to the vegetal pole of the embryos. Expressions from the fertilized egg and onwards appear to have dissipated.

4 - Discussion

Thanks to inhibition of transcription, we found that the nematode *P. vulgare* and the tardigrade *H. dujardini* require zygotic genome activation for further development after the fourth and fifth cleavage cycle respectively.

ISH was attempted in both the brachiopod *Terebratalia transversa* and the tardigrade *Hypsibius dujardini*. ISH was successful in the brachiopod, while ISH in the tardigrade posed several obstacles.

4.1 - Transcriptional inhibition and timing of the MZT

The results from the inhibition of transcription by alpha-amanitin treatment indicates that ZGA is essential for further development around the fourth and fifth cleavage cycle in *Pontonema vulgare* and *Hypsibius dujardini* respectively. Note that Figure 1.2 shows a period in time wherein maternal products degrade, and zygotic transcription escalates. Figure 4.1 does the same, while the results from our transcriptional inhibition experiments are rather a point in time when ZGA is required for further development, as the experiments only show a fraction of the whole event.

In the nematode *P. vulgare* the cell cycle was arrested after the fourth cleavage, in all but one of the embryos. This is comparable to similar experiments in *Caenorhabditis elegans* where the cell cycle was arrested after the fifth cleavage [14]. As the two species are both nematodes, and show similar features of embryogenesis this is not surprising.

In the tardigrade *H. dujardini* the cell cycle was arrested after the fifth cleavage. As there are no comparable experiments performed within the taxa of the Tardigrada, one has to make comparisons to their Ecdysozoan relatives. As seen above, these results are comparable to the findings within the two nematodes. Comparing the timing of the event to other arthropods such as the fruit fly is somewhat more complicated.

All early development within the fruit fly, characterized by the syncytium, is vastly dissimilar to all but its closest relatives. The MZT occurs, in the broadest sense, even earlier and later, across a large timespan and several cleavage cycles. The majority of the changes however occur somewhere between the eighth and fourteenth cleavage cycles. The specialized early development and the timing of the MZT in the fruit fly invites no comparison, it is however easier to compare the results of alpha-amanitin treatment in *H. dujardini* to the nematodes as the timing, of transcriptional inhibition experiments, are similar.

The sonication treatment carried out on these embryos may be seen as excessively harsh, but it is required. Alpha-amanitin would not penetrate the eggshell of the embryos, as the first run of alpha-amanitin treatment generated not mitotically arrested embryos, but rather juveniles, as the inhibitor did not penetrate the eggs. Neither did staining of the eggs by DAPI, propidium iodide, antibody staining and Hoechst have any effect. A mix of chitinase and chymotrypsin similar to the one described in section 2.9.2 was attempted, but eventually disregarded as optimizations had to be made, in addition to the treatment being quite severe on the eggs. Sonication was thus deemed the easiest and most reliable treatment to permeabilize the eggs.

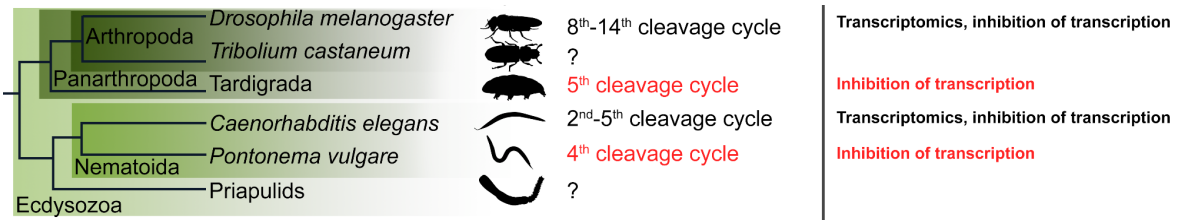

Figure 4.1 - Transcriptional inhibition within the Ecdysozoa

Tree showing select species of Ecdysozoa and their MZT timing. Known timings and methodology used from model systems are written in black. Experimental procedures performed by inhibition of transcription with alpha-amanitin treatment in the nematode *Pontonema vulgare* and the tardigrade *Hypsibius dujardini* are shown in red. Note that results shown in red indicate solely when ZGA is required for further development of the embryo, and not the period of time in which the MZT occurs as a whole as the timings shown in black.

To further the investigation into the timing of the event many more techniques could be employed. As mentioned in section 1.1.2 several studies have looked into early development of the embryo and the MZT. Some papers are based on the studies of factors required for degradation of maternal products, such as Smaug in *Drosophila* [24], studies into functionally similar homologs within the tardigrade *H. dujardini* and the nematode *P. vulgare* could greatly increase our understanding on the subject.

Other papers have focused on quantifying the degree to which maternal products are degraded and zygotic genes are activated. Many of these are based in microarray based gene profiling [9], and others by microarray hybridization in combination with advanced bioinformatics. These, and many more such techniques and inquiries could be made into the *H. dujardini* and *P. vulgare* to investigate the timing of the event further.

4.2 - Possibility of qPCRs to measure gene expression in *H. dujardini*

Embryos of *H. dujardini* were sampled procedurally from 0 hpl laying to 8 hpl. The intent behind this sequential sampling was to eventually construct cDNA libraries and to eventually lead to a qPCR in which genes of interest, both maternal and zygotic, were evaluated according to levels of relative expression. This, in coordination with the transcriptional inhibition, could also point towards a given gene being expressed either maternally or zygotically, depending on the timing. It also would have allowed us to more easily visualize the differential gene expression of zygotic and maternal genes as more advanced methods used in other papers require quite in-depth transcriptomics and bioinformatics. Unfortunately this was not to be due to time constraints and unforeseen issues regarding the work in the tardigrades.

4.3 - ISH in *T. transversa* yielded somewhat comparable results to other model species

ISH in the brachiopod Terebratalia transversa seem successful.

The expression of beta-catenin can be observed in the dorsal regions of oocytes and mature eggs in *Terebratalia transversa*. In *Xenopus,* beta-catenin can be detected as late as the blastula stage [110], in *Terebratalia* however the signal dissipates much earlier. This is unexpected, as the wnt pathway, of which beta-catenin belongs to, is so deeply conserved across Bilateria [111].

Pumilio expression can be detected throughout the cytoplasm of all stages of *Terebratalia* incorporated in this ISH. Pumilio has been known to be expressed in embryos of *Drosophila,* mice [112]*,* in germ line stem cells in *C. elegans* and human embryonic stem cells [113]. The gene is essential for maintenance and establishment of the germline across the metazoa [114]. Perhaps the mRNA for pumilio is distributed uniformly but only translated in the germline as it is in *Drosophila* by Rbfox [115]. Possibly, by way of a double ISH of pumilio and a homolog of the *Drosophila* Rbfox one could surmise the localization of pumilio. Another option would be to produce transgenic GFP-Pumilio animals, which is an extensive and costly process.

Gurken, like pumilio, can be detected throughout the cytoplasm of all stages of *Terebratalia* incorporated in this ISH. During early oogenesis in *Drosophila* the gurken mRNA can be detected in the posterior pole of the egg chamber, and in the anterodorsal corner in later

stages [116]. The localization of the gurken mRNA in *Drosophila* is tightly regulated by other genes [117] and by its 5´ and 3´ UTRs [69].

Bruno can be detected throughout the cytoplasm of all stages of *Terebratalia* incorporated in this ISH. The protein is known to repress both oskar and gurken in *Drosophila*, which are expressed posteriorly, the expected expression pattern would then be all through the cytoplasm but the posterior. The mRNA and protein has also been found to localize in the germ plasm of zebrafish [118]. Unfortunately no such localized expression pattern can be found in *Terebratalia*. The bruno-gurken interaction described in *Drosophila* has not been found in other species, and may be a system specific phenomenon. Possibly the patterns as described above are not found because they have not yet occured. Comparing so distantly related species poses obstacles of all kinds, and duplication of such specific patterns may be a lost cause. As the ISH showed uniform mRNA expression across all stages, qPCR or western blot could provide more details about expression levels of the mRNA or protein over a timeline.

All in all, the ISH performed in *Terebratalia transversa* appears a cautious success. All probes show localized expression in the egg stages, but eventually form a uniform expression level throughout the entire embryo. Many expected patterns found in other systems are not to be found within the brachiopod, such as the later dorsal-anterior localization of gurken, the all but posterior expression of pumilio as seen in *Drosophila,* the case of bruno which is found throughout embryos with the exception of the posterior or the lack of beta-catenin expression in stages later than the mature egg. This casts a slight doubt to the degree of how functionally homologous the *T. transversa* genes are to other known model species. Nonetheless, localized signals are found as to be expected, and replication of the ISH complemented with staged between the 4-cell stage and the late blastula might prove further insight into the expression of these mRNAs.

4.4 - Reflections on ISH in *H. dujardini*

As the standard protocol for ISH as described for *Terebratalia* in 2.8.1 yielded no results after several attempts, a protocol derived from [109], thoroughly described in 2.8.2 was attempted in its stead. The protocol raised complications however, at the point of separating the embryos from their eggshells by way of a 0.25G needle. Given more time to perfect and optimize the protocol, the efficiency may have been improved, as this step requires precision down to the millimeter.

Nonetheless, the genes chosen for the ISH seem to be promising as the majority of them are required for establishing AP-DV identities in the early embryo, several of them expressed either maternally or zygotically as preliminary transcriptomic data from our lab indicate, or as seen in the plethora of model species, and that the majority appear to nestle within gene trees constructed from the target gene and homologs as seen section 3.2. The exception to this is the tardigrade Jagged1a (Fig 3.2C) as it groups separately from its Ecdysozoan counterparts, meanwhile the human Jagged is nestled within the genes of the Ecdysozoa. The apparent outgrouping of this gene is unexpected and raises questions about supposed functionality not only it inself, but also in the downstream Sno of the Notch pathway.

The standard protocol not being applicable to this species may be due to a multitude of reasons. As previously mentioned, the eggshell of the *H. dujardini* is quite tough. In the standard protocol, where the eggshell is not removed, the affect of fixation might increase the toughness of the eggshells, as fixation creates covalent bonds between proteins. The following Proteinase K treatment performed during ISH aims to dissolve these bonds. As the species have not been performed ISH on in the lab, optimization is required, and prolonged Proteinase K treatment might be beneficial. On the topic of permeabilization, in the CSHP protocol the embryos were treated with a mixture of chitinase and chymotrypsin prior to addition of probes and visualizing. As the protocol met obstacles prior to visualization, assessing the efficiency of the chitinase/chymotrypsin treatment would be speculation. Nonetheless, given that the protocol has proven fruitful in the source material, the potential of the technique is high. Possibly, a combination of both Proteinase K and chitinase/chymotrypsin treatment could be optimized, if need be.

Another shortcoming is the one of human error. In the standard protocol, many embryos were lost during the process of several washes, as the embryos are as small as 25 µM and relatively translucent, they are susceptible to being glanced over and removed inadvertently. In a trial run of the CSHP protocol performed on adults, the degree to which the sample size shrunk over the course of the steps was permissible.

On the whole, ISH in the tardigrade was a mild success, not by absence of successful ISH but rather the insights the failed ISH provides. The lack of results is rather a testament to the difficulties that must be overcome when working with the species, especially in concern to embryos. Given further optimization of the technique, and further investigation into the genome the possibilities are endless to explore both the MZT and the similarities to its Ecdysozoan relatives.

4.5 - Conclusion and future prospects

Brachiopoda, one of the less studied taxa, has proven valuable to current research [103]–[105]. In this study it was used only briefly as a medium to perform ISH on known maternal axis-specifying genes in *T. transversa*. In order to broaden the understanding of the MZT and early development of the species, one could broaden the scope of genes to research in ISH, such as even more maternal determinants, early zygotic transcripts and more germline specifying genes. Even with the genes already used in this study, the knowledge of their expression and interactions could be expanded upon by additional methods.

Apart from studies into the MZT and early development, *T. transversa* could serve to explore other fields. As they are relatively easy to handle, to collect and are less understood than the average model system, the species could serve as a model species for the Brachiopoda and Spiralia. This is however limited by the difficulties of keeping them cultured, which is true for most of these clades anyhow.

As previously discussed, the taxa of Tardigrada is largely overlooked in most fields of studies. Provided optimization, the possibility of performing ISH in the tardigrade could be a powerful technique for expanding on the knowledge of itself, and the MZT. The genes chosen could have provided insight not only into the MZT but also if the pathways found in the *Drosophila* and *C. elegans* could also be found in the tardigrades, as has been done for so many other species. This, in conjunction with qPCRs for the same genes could have proved valuable insight into the topic of the MZT and early development in *H. dujardini*.

ZGA is required for further development in *P. vulgare* and *H. dujardini* after the fourth and fifth cleavage cycle respectively. In order to better understand the timing of the MZT in both *H. dujardini* and *P. vulgare* further experiments must be performed. The findings so far are only a fraction of the puzzle, thankfully several powerful techniques are already established in model systems which may be transferable to these species.

Interestingly, both the results from the transcriptional inhibition and the gene trees constructed, for these few genes, could hint at a closer tardigrada-nematoida relationship than thought. Given the miniscule amount of data collected however, this claim is largely substantiated, but could provide an interesting angle to continue exploring.

As posed by Gabriel et al. [75] the tardigrades could prove an invaluable source for studies into the evolution of development as they are so closely related to the two iconic model species *Drosophila melanogaster* and *C. elegans*. In part, this hypothesis has already proven true as it was understood that the stunted body plan of tardigrades evolved due to a loss of certain Hox genes [108]. Could the information and techniques of *C. elegans* and *Drosophila* be applied extensively to the tardigrades and other Ecdysozoans and less studied taxa, data on evolution and development within such a diverse superclade could be furthered massively. The tardigrades pose an interesting candidate for the establishment of a new model species, which is uniquely positioned within the Ecdysozoa and characterized so uniquely by its own attributes.

5 - References

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6 - Appendix

Table 6.1 - Tardigrade primer sequences and expected lengths of products

NAME	Abbr.	Forward	Reverse	Length
Held out wings	How	CACAGCTCCAACGACACTCT	TCCATCAACTGCCGCTTCTT	683
Sog/chordin	Sog	CGACTGTTTTTGCTTGCGGA	CTTATCAACTCCGCGCTTGC	2220
Bicaudal C like	BicC	GATATCTGTGCGTCGCTGGA	GGCTTCCATGTTCCAGAGCT	1472
Lethal 2, giant larvae like 2	L2GL2	CAAGAACACCACCGCCTTTG	TAGGCCAACGACTGTGTTCC	753
Lethal 2, giant larvae like 1	L2GL1	GTCATCGCGATTGGAACACG	CCTTCCAAGTGTCTGCGTCT	1482
BMP receptor type 2	BMPRII	AATCTACCCCGATCGTTCGC	GACGGAGGAGCGATCGAATT	1215
Strawberry notch	Sno	CATCAACCGCAGCAACAACA	GCAGAGATTTTTCGCGCGAT	1098
Frizzled-7	Frz7	GTCTACAACACCGTCAGCCA	GCAAAGTGGTTGGTCGTTCC	957
Jagged 1a	Jag	GGCGAATGCAGATGTCGTTC	GTTGTTCCAGTACGGGTCGT	1113
Hunchback	Hb	TGAACTTCCCGCCTGAGAAC	CCGATCGTCATCATCCGTGT	575
Toll	Tl	CTCAGACGCCTTTCCAACCT	GTTCTCCGTCAAGCCCTTCA	804

Jaworskis media:

To make Jaworski's medium:

1. Autoclave 1 L MilliQ.
2. Add 1 mL of each of the stock solutions.
3. Control and adjust the pH. It should be between 7.6 and 8.

Stock solutions for Jaworski's medium

To make stock solutions for Jaworski's medium dissolve the following in 100 mL in MilliQ.

Product	Quantity per 100 mL
Ca(NO3)2*4H2O	2.0 g
KH2PO4	1.24 g
MgSO4*7H2O	5.0 g
NaHCO3	1,59 g
EDTAFeNa, EDTANa2	0.225 g, 0.225 g
H3BO3, MnCl2*4H2O, (NH4)6Mo7O24*4H2O	0,248 g, 0,139 g, 0,10 g
Cyanocobalamin, Thiamine HCl, Biotin	0,004 g, 0,004 g, 0,004 g
NaNO3	8.0 g
Na2HPO4*12H2O	3.6 g