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Research Article

Monitoring for propagules of *Ciona intestinalis* in marine water samples: the development of temporal gene expression markers for viability and life stage specific assays

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Abstract

Due to the challenges of managing invasive species, new technologies to assist in monitoring for the early detection of early life stages of invasive species is critical for many regions of the world. Invasive tunicates have been causing challenges to the shellfish aquaculture industry in Atlantic Canada, with *Ciona intestinalis* causing the most significant challenges to date. While existing early detection systems for this species depend on morphological screening of preserved water samples or DNA based assay screening of fresh/preserved water samples, these methods are limited as they do not have the ability to evaluate the viability of propagules present, and they are also not able to distinguish between different life stages of *C. intestinalis* that may be present in water samples. These factors are important since non-viable propagules are not a risk to shellfish regions and unfertilized eggs have a different risk factor than viable free swimming larvae. It is most important for shellfish aquaculture growers to know when free swimming larvae life stages are present in the water so that they can appropriately time their mitigation treatments. This study successfully used RT-qPCR to screen temporally expressed genes for exclusively expressed mRNA transcripts at target life stages of *C. intestinalis*. In addition, this study identified mRNA transcripts that are quickly degraded post-mortem which makes them excellent candidate markers for larval viability assays. Based on the results of this study, HOX2 or TPM1 markers can be used in RT-qPCR assays to determine whether the *C. intestinalis* material present in a water samples is an egg or a larvae. Finally the TPM2 marker can be used to evaluate whether the larvae are viable or non-viable. Such an assay would be beneficial in screening water samples around mussel processing plant effluent outflows as well as water samples from ballast water, thereby reducing the potential further spread of the invasive species.

Key words: invasive species; tunicates; monitoring; aquaculture; molecular detection; assays

Introduction

There are currently four invasive tunicates which are causing significant fouling problems to the mussel aquaculture industry in Atlantic Canada. Of the four invasive tunicates, the vase tunicate, *Ciona intestinalis* (Linnaeus, 1767), is the species that poses the greatest threat to the mussel aquaculture industry due to the weight of fouling that this species exhibits on aquaculture lines and gear (Ramsay et al. 2008; Ramsay et al. 2009). *C. intestinalis* has become the dominant tunicate species in many areas due to its ability to reproduce at lower temperatures, its fast rate of growth, its tolerance to high density crowding and its mucoidal tunic which inhibits settlement from other tunicate species (Ramsay et al. 2008; Ramsay et al. 2009). Although Carver et al. (2003) demonstrated that *C. intestinalis* produced gametes in April at 4°C, *C. intestinalis* recruitment was not observed until mid-June at 8°C (Ramsay et al. 2008). This reveals that although gametes may be detected in water samples, this does not necessarily mean that viable larvae will also be present. To increase the effectiveness of mitigation methods, it is important for mussel aquaculture growers to know when viable larvae are present

in their aquaculture lease areas, so that they can plan treatment protocols accordingly. Current monitoring for larvae in this area is being conducted using microscope surveys of concentrated water samples. Having high throughput molecular assays that can screen water samples for invasive tunicate eggs and larvae would be beneficial as a management tool.

Molecular assays have been developed and utilized in monitoring strategies for many species of aquatic invasive species (AIS) (Willis et al 2009; Stewart-Clark et al 2013) and their utility as a management tool for ecosystem managers has been reviewed (Darling and Blum 2007; Darling and Mahon 2011). The molecular assay CIONAINTESTCOI (Stewart-Clark et al. 2009) has been used to detect the presence of *C. intestinalis* tissue in water samples collected in the field to evaluate the presence/absence of this species as well as to confirm species identifications for ecosystem managers (Stewart-Clark et al. 2013). However, this assay cannot distinguish between different life stages of this invasive species. This is due to the fact that the species assay detects DNA, which is present at all life stages of the individual tunicate (Burreson 2000). While each cell of an organism contains copies of identical DNA molecules through all stages of development, mRNA content and quantity varies throughout different stages of development (Azumi et al. 2007). Therefore to distinguish between different life stages in *C. intestinalis*, mRNA based detection assays could be developed. Such an assay could be implemented in surveillance and monitoring programs to screen for eggs and larvae of *C. intestinalis* in water samples from different coastal areas, from mussel processing plant effluent and from ballast water tanks. While other studies use RT-qPCR assays to detect infectious agents such as viruses (Cowley et al. 2009; Grajkowska et al. 2009; Huang et al. 2009; Nadin-Davis et al. 2009) and cancerous markers (Kondoh et al. 2007; Loos et al. 2010), this study marks the first to use RT-qPCR as a diagnostic assay for different life stages of an organism for the purpose of AIS monitoring.

Since the genome of *C. intestinalis* was sequenced (Dehal et al. 2002), extensive analysis has occurred measuring gene expression in *C. intestinalis* across many life stages during development (Hotta et al. 2000; Nishikata et al. 2001; Satou et al. 2002; Chiba et al. 2003; Hino et al. 2003; Ishibashi et al. 2003; Imai et al. 2004; Yagi et al. 2004; Kawashima et al. 2005; Deyts et al. 2006; Miwata et al. 2006; Yamada 2006; Azumi et al. 2007; Comes et al. 2007; Hamaguchi et al. 2007; Hotta et al. 2007; Imai and Meinertzhagen 2007; Matsumoto et al. 2007; Sekiguchi et al. 2007). From these studies, it is clear that temporal gene expression changes throughout the developmental life stages of *C. intestinalis* (Satou et al. 2002; Kawashima et al. 2005; Azumi et al. 2007). Based on EST data, Kawashima et al. (2005) showed that 25% of developmental genes in *C. intestinalis* are expressed at multiple points during development while the remaining 75% are either single use genes or genes that are constantly expressed across all developmental life stages. The main objective of this study is to screen the expression of selected genes in egg, larvae and adult stages of *C. intestinalis* with RT-qPCR in order to develop life stage specific molecular markers for the egg and larval life stages of *C. intestinalis*.

The life stage specific genes targeted in this study were selected based on fluorescence *in situ* hybridization (FISH), whole-mount *in situ* hybridization (WMISH), EST, and microarray studies involving multiple life stages of *C. intestinalis* (Yoshida et al. 1997; Chiba et al. 2003; Ikuta et al. 2004; Imai et al. 2004; Yamada et al. 2005; Miwata et al. 2006). From these studies, six genes were chosen as potential life stage markers for free swimming larvae: Homeobox-2 (HOX2) (Ikuta et al. 2004), Homeobox even-skipped homolog B(EvxB) (Ikuta et al. 2004), Actin-1 (MA1), Tropomyosin-2 (TPM2) (Chiba et al. 2003), Tropomyosin-1 and CA3 (Satoh 2000). In addition, four genes were screened as potential life stage markers for unfertilized eggs: PEM Homeobox gene (PEM) (Yoshida et al. 1997), PEM-13 Homeobox gene (PEM-13) (Yamada et al. 2005), Zinc Finger 364 (ZF364) and Zinc Finger 054 (ZF054) (Miwata et al. 2006).

An integral component of RT-qPCR assays are normalisation genes that act as internal controls across all experimental variables (Bustin 2002). Normalisation genes are used to correct for variation in gene expression which may exist within the experimental procedure such as between individual samples, different reagents, extraction procedures, presence of inhibitors and inefficiencies in assay runs. If assays have appropriate internal controls, treatment effects can be detected despite any variation due to experimental effects. A defining characteristic of appropriate normalisation genes is that they must exhibit stable gene expression across all

variables within each experiment. Since numerous studies have now shown that no one gene is suitable as a universal normalisation gene across all possible experimental conditions, appropriate normalisation genes must be validated in each experimental situation (Bustin et al. 2005). In addition, qRT-PCR assays must no longer involve only one normalisation gene, but instead include several normalisation genes (Vandesompele et al. 2002; Bustin et al. 2005; Nolan et al. 2006; Peters et al. 2007). Several normalisation genes have been used in other qRT-PCR experiments involving *C. intestinalis* including RPS18, RPL11, (Olinski et al. 2006) RPS27A (Olinski et al. 2006; Comes et al. 2007), GAPDH (Olinski et al. 2006; Coric et al. 2008) calmodulin (CiCAM) (Piscopo et al. 2000; Matias et al. 2005) and α-tubulin (DeLigio and Ellington 2006). The second objective of this experiment is to screen the gene expression levels of these six normalization genes to determine which exhibit stable gene expression across the life stages involved in this study. In this study, normalisation genes will be used as positive controls rather than normalisation factors, since gene expression levels are not being compared across the three life stages. Instead, genes are being screened for exclusive expression at each life stage, so although no expression may be seen for the target gene at all other life stages, the expression of normalisation genes will indicate that this is due to the fact that the target transcript is not present in the sample and not that the sample has degraded.

As indicated above, the target molecule for the life stage specific assays developed in this study are mRNA transcripts. mRNA transcripts have varying levels of stability depending on the gene, with some mRNA transcripts degrading within minutes of transcription and others remaining stable for up to 24 hours (Tourrière et al. 2002). The stability of an mRNA transcript is often related to the duration that its translated protein is required in the cell. Proteins that are quickly produced in response to developmental or external cues are often from mRNA transcripts with short half-lives whereas proteins that are present in stable concentrations over a long time period are generally from mRNA transcripts with long half-lives (Guhaniyogi and Brewer 2001). For gene regulation purposes, mRNA transcripts are often degraded by endonuclease and exonuclease activity (Tourrière et al. 2002). In addition post-death, mRNA is rapidly degraded by RNases (Fontanesi et al. 2008). This characteristic may allow for some mRNA transcripts with short post-mortem half-lives to be used as viability assays in *C. intestinalis* larvae. The degradation of mRNA transcripts post-mortem has been the focus of many recent studies. These studies have shown that mRNA degradation post-mortem is gene dependent, with some mRNA transcripts degrading within minutes of death, and others remaining stable after 48 hours (Zhao et al. 2006; Fontanesi et al. 2008) and even 96 hours (Yasojima et al. 2001). It would be important for surveillance and monitoring programs, especially in programs screening ballast water or mussel processing plant effluent water, to be able to determine whether the larvae being detected in water samples are viable or non-viable organisms. This could be determined by targeting mRNA transcripts that are quickly degraded post-mortem. The third objective of this study is to evaluate the transcript stability of life stage specific markers generated in this study to determine whether any of the target genes could be markers for larval viability.

Materials and methods

Tissue collection

Ciona intestinalis samples were collected from Murray River Prince Edward Island (PEI), from July to August 2007. Eggs were collected by piercing the oviduct with a syringe needle and eggs were removed using a pipette. Eggs were immediately placed in 1.5ml tubes containing 200µl RNAlater and were incubated overnight at 4^{o}C. Each tube contained pools of eggs from 3 individuals. Tubes were then centrifuged at 2000 × g for 2 minutes and RNAlater was removed from the tubes with a pipette. The tubes containing eggs were then snap frozen in liquid nitrogen and stored at -80^{o}C.

Free swimming larvae were generated in the lab by removing egg and sperm samples from multiple *C. intestinalis* individuals. These gametes were then placed in separate graduated cylinders containing 200ml of filtered sea water before being mixed together by swirling. Gametes were then poured into petri dishes with filtered sea water and left for 18 hours. Free swimming larvae were individually collected by pipette and placed in microcentrifuge tubes containing between 111–364 free swimming larvae. Tubes containing free swimming larvae were immediately snap frozen in liquid nitrogen and stored at -80^{o}C. Free-swimming larvae were not placed in

RNAlater due to the results of preliminary experiments which showed that the larvae float on top of the RNAlater solution and do not pellet making later removal of the RNAlater solution from the free-swimming larvae impossible.

Three adult specimens were used to collect adult tissue. The tunic was first removed from each specimen via dissection with a scalpel blade. Individual specimens were then finely chopped with a scalpel blade and tissues were mixed together to ensure that each sample contained multiple adult tissues. Tissue mixtures from each individual were divided into four 30 mg quantities for later RNA extraction and were placed in 1.5ml microcentrifuge tubes. Adult samples were then snap frozen in liquid nitrogen and stored at -80^{o}C until RNA was extracted.

RNA Extractions

All RNA extractions were performed on egg, free swimming larvae and adult tissues using Qiagen RNeasy Minikits (Qiagen Inc., Canada). Extractions were all performed following manufacturer's instructions. For each of the three adult specimens, RNA from all four tubes per individual was pooled prior to cDNA synthesis so that RNA from the entire individual would be available for each of the three adult cDNA synthesis.

cDNA Synthesis

cDNA synthesis was performed using 100 ng RNA from each sample. Qiagen QuantiTect RT kits were used for all cDNA synthesis reactions and all reactions were conducted according to manufacturer's protocol.

qPCR Primer Design

All life stage specific primers were manually designed from EST sequences obtained from GenBank and from Ghost Database (http://ghost.zool.kyoto-u.ac.jp/indexr1.html) and JGI *Ciona intestinalis* V1.0 (http://genome.jgi-psf.org/ciona4/ciona4.home.html) and from ANISEED (http://crfb.univ-mrs.fr/aniseed/) (Table 1). To minimize false-positives, all primers were assessed to ensure specificity by NCBI-BLAST (National Center for Biotechnology Information- Basic Local Alignment Search Tool) (Altschul et al. 1997). Primer suitability was further evaluated using IDT Oligoanalyzer 3.0. Normalisation gene primers were all obtained from other RT-qPCR studies involving *C. intestinalis*: RPS18, RPL11, (Olinski et al. 2006) RPS27A (Olinski et al. 2006; Comes et al. 2007), GAPDH (Olinski et al. 2006; Coric et al. 2008) calmodulin (CiCAM) (Piscopo et al. 2000; Matias et al. 2005) and α-tubulin (DeLigio and Ellington 2006) (Table 2).

qPCR

Quantitative analysis of RNA levels was performed using iTaq *SYBR Green* Supermix with *ROX* (Bio-Rad, Canada) on a Chromo4™ Real-time detector (Bio-Rad, Canada). Each sample was run in duplicate or triplicate and average Ct values were calculated for each sample. Every sample was run in three serial dilutions to ensure that reaction efficiencies were appropriate. Two normalisation genes (RPL11 and RPS27A) were monitored in every run to ensure that target sample levels were not significantly altered during extraction, cDNA synthesis and amplification procedures. No template controls were included in each run to ensure levels were not impacted by contamination. No-RT controls were run with most samples to ensure that samples were not contaminated with genomic DNA. Ct values for each target gene were only compared to samples run in the same run to reduce between run variations in fluorescence levels. Melting curves were calculated and evaluated in every run to ensure that primer dimers were not contributing to fluorescence levels. Normalisation genes were screened using samples from all adult and free swimming life stages and results were analyzed using BestKeeper-1 (Pfaffl et al. 2004).

Transcript Stability Analysis

Free swimming larvae were generated as previously described. Free swimming larvae were then placed in petri dishes filled with fresh water. Previous experiments determined that 2 minute exposures to fresh water were sufficient to kill free swimming larvae of *C. intestinalis*. Following the 2 minute exposure, 100 larvae were sampled and immediately snap frozen in liquid nitrogen. 100 larvae were then sampled and snap frozen at 30 minutes post exposure, 1, 2, 4, 8, and 24 hour periods post exposure. RNA was then extracted and cDNA synthesized as described above. qPCR was performed as previously described. Ct levels were compared to evaluate whether there was a change in transcript levels at the different post-mortem intervals.

Table 1. qPCR primers designed for life stage specific assays for *Ciona intestinalis* eggs and larvae.

Primer Name	Sequence 5'-3'	GenBank Accession Number Or JGI *C. intestinalis* V1.0 region
MAF	CTGCTGGAATCCACGAG	AK115759
MAR	GGTGGACAATAGATGGGC	AK115759
CA3F	GAAAGGAGGGTTTCAGGAG	XM_002129028
CA3R	GATCCTCCAGCAAGAACG	XM_002129028
TPM1F	AGGACCCAGACACTTTGG	NM_001032538
TPM1R	CCCAGTAGCTTTGTCTTCG	NM_001032538
TPM2F	GAATTGGAATCTGCCCAAG	JGI *Ciona intestinalis* v1.0 Scaffold_22:380810-383651
TPM2R	TATAAACCCCAATCCCACC	JGI *Ciona intestinalis* v1.0 Scaffold_22:380810-383651
HOX2F	GGCGCATCCAGGAGACTACG	AB210494
HOX2R	CGTCGGCGCTTGTTACGTCAG	AB210494
ZF054F	CGCATCACTCTTACATCCACATCAATGAGA	XM_002130176
ZF054R	TTTACTCCTGTATTGTCAAACTCCCCATT	XM_002130176
ZF364F	GGTGGAAGCAAAGGTCTGCAC	AK115172
ZF364R	GCACCATTATCCCTCAATCCCCTGG	AK115172
EvxBF	TAGAACTCTACATCTCTGTCGCACCT	AK174659
EvxBR	CGAACTCTGCTATGTAGTACAAACTGGTAC	AK174659
PEMF	GACCTCGACCCCGCAGAG	AK113383
PEMR	TAGCGGTCACACGGCGTG	AK113383
PEM13F	GTGCGGCAATCTGATGCGAGG	BW275407
PEM13R	CTTTGGGTGTGAAGGGCAGTT	BW275407

Table 2. qPCR primers screened as normalization genes for life stage specific assays.

Primer Name	Sequence 5'-3'	Reference
RPS18F	GAATCGGCCGCAGGTTTAG	Olinski et al. 2006
RPS18R	CCGGCGCGTTTCGTAA	Olinski et al. 2006
RPS11F	GTTCGATACCGCCGAAAAAA	Olinski et al. 2006
RPS11R	CATGCCATCAGGCTTGGTTAG	Olinski et al. 2006
α-TubulinF	TGAGCCCTACAACTCCATCC	DeLigio & Ellington 2006
α-TubulinR	CAAAGCACCATCGAATCTCA	DeLigio & Ellington 2006
RPS27AF	GAATCGGCCGCAGGTTTAG	Olinski et al. 2006
RPS27AR	CCTTCCTTATCCTGAATCTTTGCT	Olinski et al. 2006
CAMF	GTTGATGCTGACGGCAACG	Piscopo et al. 2000
CAMR	TCAATCAGCCTATGGAATGA	Piscopo et al. 2000
GAPDHF	GCACTCGTACACTGCTACCCAGAAGAC	Olinski et al. 2006
GAPDHR	GCTGTATCCAAATTCATTGTCGTACCAG	Olinski et al. 2006

Results

Normalisation Gene Screening

Of the six genes screened as normalisation genes in *C. intestinalis* adults and free swimming larvae, only two were selected by BestKeeper as appropriate normalisation genes: RPL11 and RPS27A (Figure 1). The gene expression of all other candidate normalisation genes was too variable (data not shown) and had CP standard deviations > 1.0 which is the cut-off for genes in BestKeeper.

Life Stage Marker

Egg Life Stage

Gene expression of PEM and PEM-13 was detected in all unfertilized egg and free-swimming larvae samples (Figures 2 and 3). ZF364 gene expression was detected in all unfertilized egg samples and in 2 out of 5 free swimming larvae samples (Figure 4). ZF054 gene expression was not detected at any of the life stages in this study although the two normalization genes RPS27A and RPL11 were detected in all samples.

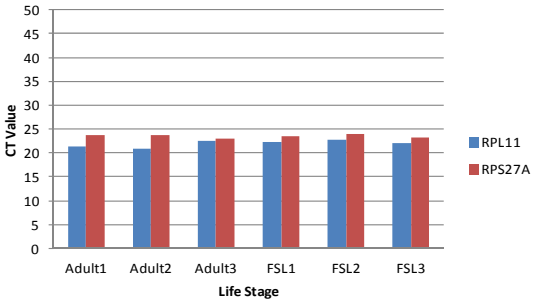

Figure 1. Gene expression levels of RPL11 and RPS27A genes in adult and free swimming larvae (FSL) life stages of *Ciona intestinalis*.

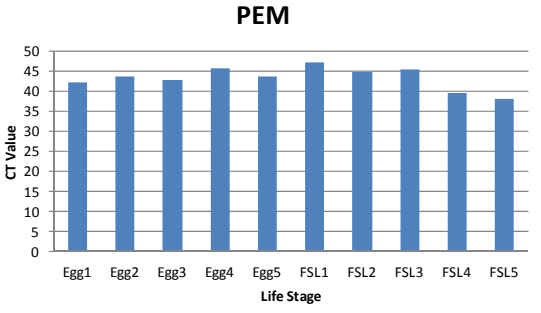

Figure 2. Gene expression of PEM at egg and free swimming larvae (FSL) life stages of *Ciona intestinalis*.

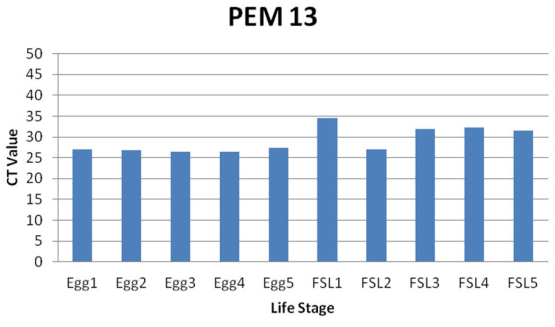

Figure 3. Gene expression of PEM13 at egg and free swimming larvae (FSL) life stages of *Ciona intestinalis*.

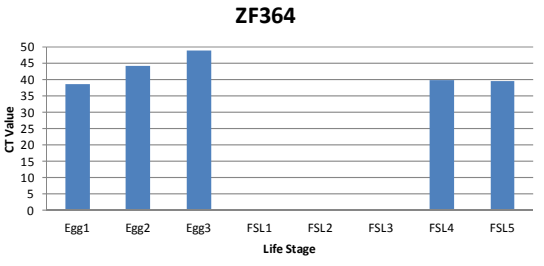

Figure 4. Gene expression of ZF364 in egg and free swimming larvae (FSL) life stages of *Ciona intestinalis*.

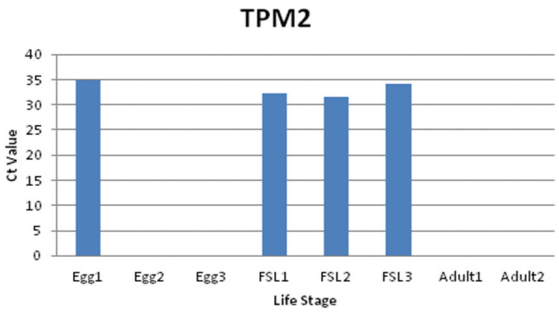

Figure 5. Gene expression of TPM2 gene in egg, free swimming larvae (FSL) and adult life stages of *Ciona intestinalis*.

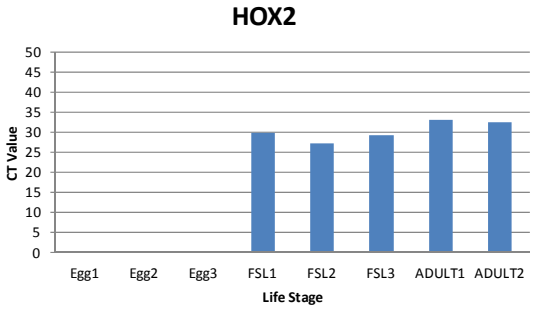

Figure 6. Gene expression of HOX2 gene in egg, free swimming larvae (FSL) and adult life stages of *Ciona intestinalis*.

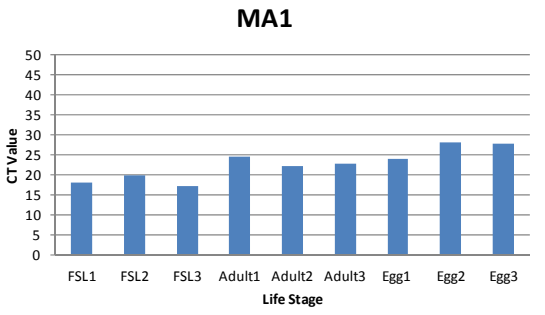

Figure 7. Gene expression of MA1 gene in free swimming larvae (FSL), adult and egg life stages of *Ciona intestinalis.*

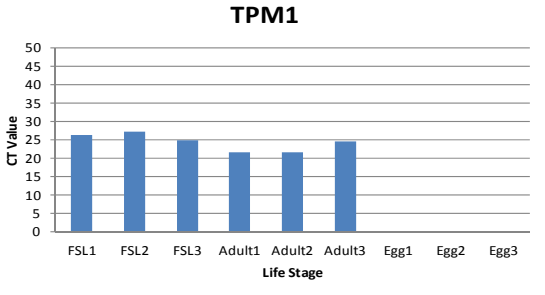

Figure 8. Gene expression of TPM1 gene at free swimming larvae (FSL), adult and egg life stages of *Ciona intestinalis*.

Free swimming larvae life stage

TPM2 gene expression was not detected in adult samples, but was detected in every free swimming larvae sample (Figure 5). TPM2 gene expression was also detected in one of the unfertilized egg samples. HOX2 gene expression was detected in all free swimming larvae and adult samples, but was not detected in any of the egg samples (Figure 6). MA1 gene expression was detected in every sample across all three life stages (Figure 7). TPM1 gene expression was detected in free swimming larvae and adult life stages, but was not detected in any of the egg samples (Figure 8). CA3 expression was detected in every sample across all three life stages (Figure 9).

Transcript Stability Experiment

Both HOX2 and TPM1 gene expression remained stable 24 hours post-mortem (Figure 10-11). TPM2 gene expression was last detected 4 hours post-mortem (Figure 12). In all three cases, normalisation gene expression (RPS27A and RPL11) remained stable 24 hours post-mortem.

Discussion

Normalization Gene Analysis

As recommended by Bustin et al. (2005) we screened multiple normalisation genes and validated two that exhibited stable gene expression across multiple life stages in *C. intestinalis*: RPS27A and RPL11. These two genes were also validated as appropriate normalisation genes in a previous study involving *C. intestinalis* (Olinski et al. 2006). However four other normalisation genes that were used in other studies involving *C. intestinalis,* RPS18 (Olinski et al. 2006), GAPDH (Olinski et al. 2006; Coric et al. 2008) calmodulin (CiCAM) (Piscopo et al. 2000; Matias et al. 2005) and α-tubulin (DeLigio and Ellington 2006), were too variable to be suitable as normalisation genes in this study. These findings underscore the importance of screening normalisation genes within each experimental situation instead of assuming that normalisation genes deemed appropriate in one study can be used universally in others. For this study, our normalisation genes were used as positive controls instead of normalisation genes, since we are screening life stage genes for presence/absence of gene expression and not comparing gene expression levels across different samples.

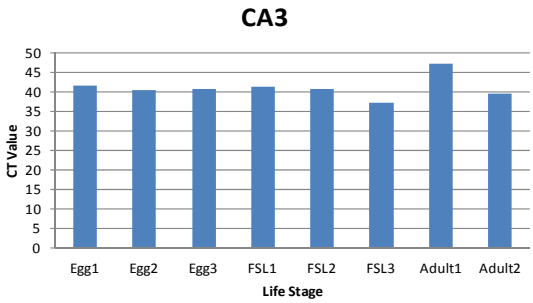

Figure 9. Gene expression of CA3 gene in egg, free swimming larvae (FSL) and adult life stages of *Ciona intestinalis*.

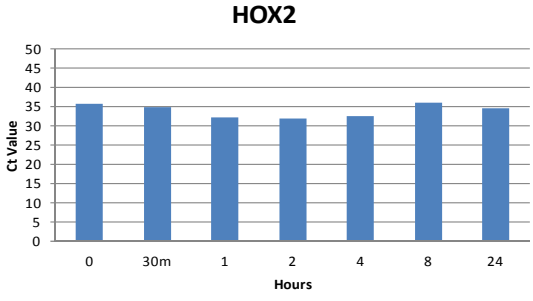

Figure 10. Transcript levels of HOX2 detected after 0-24 hours post mortem in *Ciona intestinalis* free swimming larvae.

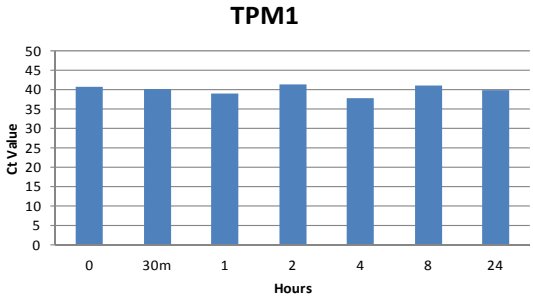

Figure 11. Transcript levels of TPM1detected after 0-24 hours post mortem in *Ciona intestinalis* free swimming larvae.

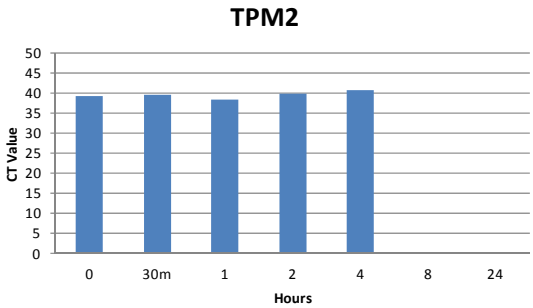

Figure 12. Transcript levels of TPM2 detected after 0-24 hours post mortem in *Ciona intestinalis* free swimming larvae.

Life Stage Analysis

Free swimming larvae

The temporal specificity of the TPM2 gene, as shown through the RT-qPCR results, makes it an excellent candidate as a marker for the free swimming larvae life stage of *C. intestinalis*. The free swimming larva is the only motile life stage of *C. intestinalis* and as such contains specific isoforms of muscle that are present in the larval tail but which are lost during metamorphosis. The muscles that make up the adult heart and body wall consist of different isoforms of muscle protein (Chiba et al. 2003). EST studies have shown that TPM2 is one muscle gene that has shown to be exclusively expressed from the cleaving embryo to the larval life stage (Satoh et al. 2000; Chiba et al. 2003). These RT-qPCR results confirm this temporal specificity with expression detected in all free swimming larvae samples but not in any adult specimens. There was one egg sample that exhibited TPM2 expression; however the other pools of eggs did not, making contamination a possible source for this unexpected expression of the TPM2 gene. An RT-qPCR assay that can reliably distinguish free swimming larvae from other life stages of *C. intestinalis* is critical for aquaculture industry managers to determine when tunicates are reproductively active and thus posing a risk to aquaculture gear and mussel lines. Two other genes, HOX2 and TPM1 are also potential markers to distinguish between egg and free swimming larva life stages in water samples since our RT-qPCR results have shown that these transcripts are not present in egg samples but were always detected in free swimming larvae samples. This temporal specificity was also exhibited in fluorescence *in situ* hybridization (FISH) and whole-mount *in situ* hybridization (WMISH) experiments spanning egg to juvenile life stages, in which Ci-HOX2 was only detected during the larval-like stage within trunk cells (Ikuta et al. 2004). In this study, these transcripts were also present in adult samples. However, *C. intestinalis* adults are sessile and remain attached to hard substrates for the remainder of their life cycle. As such, they are not normally present in the water column where sampling for eggs and larvae occur.

Unfertilized Egg

All of the genes targeted in this study as egg specific markers (PEM Homeobox gene, PEM-13 Homeobox gene, Zinc Finger 364 gene and Zinc Finger 054 gene) showed gene expression in all three life stages. In previous studies, no expression of ZF054 and ZF364 were detected beyond the fertilized eggs stage with expression signals disappearing before the 16 cell stage (Miwata et al. 2006). Yamada et al. (2005) found PEM and PEM13 gene expression in the fertilized egg and the 8 cell stage embryo, however zygotic expression of this transcript was not detected (Yamada 2006). Other studies have shown that qRT-PCR is more sensitive than WMISH in detecting mRNA transcripts (Meyers-Wallen 2003; Thermes et al. 2006) which may explain the incongruence between our results and those of previous studies based on WMISH data. The present study was unable to find an unfertilized egg specific life stage marker for *C. intestinalis*. This life stage was particularly difficult to find potential target genes to screen for since so many of the transcripts present in eggs are maternally transcribed and therefore present in adults as well as egg life stages (Azumi et al. 2007). Nomura et al. (2009) conducted a proteomic analysis of three life stages of *C. intestinalis* (unfertilized egg, 16-cell embryo, tadpole larvae) in which they discovered 5 proteins that were uniquely expressed in the unfertilized egg. Two of these proteins could not be classified while the three others were closely correlated to proteins found in other organisms including: Glutamic-oxaloacetic transaminase 2b (GOT2), ATPase family, AAA domain containing 3A (ATAD3A), Isovaleryl Coenzyme A dehydrogenase (Nomura et al. 2009). Although Nomura et al. (2009) did not evaluate the expression of these three proteins in adults, EST analysis has shown GOT2 gene expression in eggs, embryos, juveniles and young adults. Isovaleryl Coenzyme A dehydrogenase was also found in embryos and adults of *C. intestinalis* in EST analysis (Satou et al. 2004) and ATAD3A was found in eggs, embryos and adults (Satoh et al. 2000).

Transcript stability

The quick post mortem degradation rate of the TPM2 transcripts evaluated in this study (4–8 hours) makes this gene an excellent indicator of viability in *C. intestinalis* larvae. This is an important characteristic of the assay as non-viable larvae will not cause false positives if present in water samples. This is particularly important in screening mussel processing plant effluent water where water treatments are implemented to ensure

that eggs and larvae of invasive tunicates are killed prior to exiting the plant into adjacent bays. These assays could be used to ensure that the treatment programs are functioning properly and that no viable larvae are being introduced to bays from effluent outflow. This viability assay could also be used to screen and test the efficiency of ballast water treatments since non-viable larvae pose less of a risk to new regions than viable larvae. One other study (Hellyer et al. 1999) previously developed RT-qPCR assays to evaluate viability in *Mycobacterium tuberculosis* in order to rapidly evaluate drug susceptibility of specific strains.

Conclusion

Life stage specific assays based on RT-qPCR primers specific to the free swimming larvae life stage can be used to distinguish between egg and larvae of *C. intestinalis* in water samples. Such assays would be extremely useful in invasive species surveillance and monitoring programs. To distinguish between eggs, viable larvae and non-viable larvae in water samples, multiple markers could be used in conjunction with each other. The CIONINTESTCOI assay (Stewart-Clark et al. 2009) could first be used to determine that *C. intestinalis* material is present in the water samples. The HOX2 or TPM1 markers could then be used in a RT-qPCR assay to determine whether the *C. intestinalis* material is egg or larvae. Finally the TPM2 marker could then be used to evaluate whether the larvae are viable or non-viable. Such an assay would be beneficial in screening water samples around mussel processing plant effluent outflow as well as ballast water. While DNA based assays cannot distinguish viable material from non-viable, life stage specific qRT-PCR assays using transcripts with varying rates of transcript stability can be used to distinguish between viable and non-viable larvae.

It is important to note that these RT-qPCR assays have only been tested in laboratory conditions in artificial filtered seawater. Before being implemented into any surveillance or monitoring program, the assays would need to be validated in field conditions since contaminants and inhibitory compounds found in environmental water samples can impact assay sensitivity and efficacy (Tebbe and Vahjen 1993; Johnson et al. 1995; Wilson 1997; Toze 1999; Cunningham 2002).

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