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Tissue-specific evaluation of suitable reference genes for RT-qPCR in the pond snail, *Lymnaea stagnalis*

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Reverse transcription quantitative PCR (RT-qPCR) is a robust technique for the quantification and comparison of gene expression across multiple tissues. To obtain reliable results, one or more reference genes must be employed to normalize expression measurements among treatments or tissue samples. Candidate reference genes must be validated to ensure that they are stable prior to use in qPCR experiments. The pond snail (*Lymnaea stagnalis*) is a common research organism, particularly in the areas of learning and memory, and is an emerging target for qPCR experimentation. However, no systematic assessment of reference genes has been performed in this animal. Therefore, the aim of our research was to identify stable reference genes to normalize gene expression data from a variety of tissues in *L. stagnalis*. We evaluated a panel of seven reference genes across six different tissues in *L. stagnalis* with RT-qPCR. The genes included: *elongation factor 1-alpha* (*EF1α*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *beta-actin* (*ACTB*), *beta-tubulin* (*TUBB*), *ubiquitin* (*UBI*), *prenylated rab acceptor protein 1* (*Rapac1*), and a voltage gated potassium channel (*VGKC*). These genes exhibited a wide range of expression levels among tissues. The stability of each of the genes was consistent when measured by any of the standard stability assessment algorithms: geNorm, NormFinder, BestKeeper and RefFinder. Our data indicate that *GAPDH* and *EF1α* are highly stable in the tissues that we examined (central nervous system, tentacles, lips, penis, foot, mantle) as well as in pooled analyses. We do not recommend *VGKC* for use in RT-qPCR experiments due to its relatively low expression stability. Our results were generally congruent with those obtained from similar studies in other molluscs. Given that a minimum of two reference genes are recommended for data normalization, we suggest *GAPDH* and *EF1α* are a strong option for multi-tissue analyses of RT-qPCR data in *Lymnaea stagnalis*. 
Tissue-specific evaluation of suitable reference genes for RT-qPCR in the pond snail, *Lymnaea stagnalis*

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Abstract

Reverse transcription quantitative PCR (RT-qPCR) is a robust technique for the quantification and comparison of gene expression across multiple tissues. To obtain reliable results, one or more reference genes must be employed to normalize expression measurements among treatments or tissue samples. Candidate reference genes must be validated to ensure that they are stable prior to use in qPCR experiments. The pond snail (*Lymnaea stagnalis*) is a common research organism, particularly in the areas of learning and memory, and is an emerging target for qPCR experimentation. However, no systematic assessment of reference genes has been performed in this animal. Therefore, the aim of our research was to identify stable reference genes to normalize gene expression data from a variety of tissues in *L. stagnalis*. We evaluated a panel of seven reference genes across six different tissues in *L. stagnalis* with RT-qPCR. The genes included: *elongation factor 1-alpha (EF1α)*, *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, *beta-actin (ACTB)*, *beta-tubulin (TUBB)*, *ubiquitin (UBI)*, *prenylated rab acceptor protein 1 (Rapac1)*, and a voltage gated potassium channel (*VGKC*). These genes exhibited a wide range of expression levels among tissues. The stability of each of the genes was consistent when measured by any of the standard stability assessment algorithms: geNorm, NormFinder, BestKeeper and RefFinder. Our data indicate that *GAPDH* and *EF1α* are highly stable in the tissues that we examined (central nervous system, tentacles, lips, penis, foot, mantle) as well as in pooled analyses. We do not recommend *VGKC* for use in RT-qPCR experiments due to its relatively low expression stability. Our results were generally congruent with those obtained from similar studies in other molluscs. Given that a minimum of two reference genes are recommended for data normalization, we suggest *GAPDH* and *EF1α* are a strong option for multi-tissue analyses of RT-qPCR data in *Lymnaea stagnalis*. 
**Background**

*Lymnaea stagnalis* is a freshwater snail with an extensive history of proximate mechanism research. Previous studies have primarily focused on its neurobiology, as well as its endocrinology, immunology and shell morphology (Chase, 2002; Benjamin, 2008). The central nervous system (CNS) is relatively simple, with approximately 20,000 neurons, many of which are large and easily identifiable, making them amenable for studies of learning, memory, motor pattern generation, neuronal regeneration and synapse formation (Lukowiak; Elliott & Susswein, 2002; Chase, 2002; Lukowiak et al., 2003; Getz et al., 2018). Parallel investigations into the neuroendocrine (Koene, 2010; Pirger et al., 2010) and immune (van der Knaap, Adema & Sminia, 1993; Gust et al., 2013a) systems of *L. stagnalis* and other gastropods have improved our understanding of reproductive behaviour, immunity and toxicology of *L. stagnalis*.

Additionally, *L. stagnalis* has been used as a research organism to study the molecular mechanisms that guide shell formation (Boer & Witteveen, 1980; Ebanks, O’Donnell & Grosell, 2010; Hohagen & Jackson, 2013; Jackson, Herlitze & Hohagen, 2016; Herlitze et al., 2018).

A broad range of traditional techniques have been used in past studies, but modern molecular genetics have yet to be thoroughly incorporated into the study of *L. stagnalis*. Methods to study gene expression such as reverse transcription quantitative PCR (RT-qPCR) and *in situ* hybridization (among others) have been established but have not yet been used extensively. The few studies using RT-qPCR have spanned several topics including chirality, learning, and memory (Foster, Lukowiak & Henry, 2015; Davison et al., 2016; Korneev et al., 2018; Dong et al., 2018). Additionally, a handful of studies have used *in situ* hybridization in this species,
primarily in sections and whole mounts of the CNS, as well as larvae (Dirks et al., 1989; Boer et al., 1992; Croll & Minnen, 1992; Sadamoto et al., 2004). Our goal here is to continue to build the foundation for further molecular genetics studies in *L. stagnalis*, by validating candidate reference genes for RT-qPCR.

RT-qPCR is a robust technique for the quantification of the mRNA for a specific transcript. It can be used alongside *in situ* hybridization or other visualization techniques to establish patterns of gene expression in animals. For such experiments, relative quantification against one or more reference genes is the most common choice to compare gene expression across samples (Schmittgen & Livak, 2008; Ruijter et al., 2009). Historically, highly expressed cellular maintenance genes have been haphazardly selected as reference genes for qPCR experiments in many species because expression of such genes was thought to be inherently stable (Kozera & Rapacz, 2013). However, many of these genes have been shown to have unstable expression in several species of plants and animals, highlighting the importance of species-specific validation prior to use (Dheda et al., 2004; Barber et al., 2005; Jonge et al., 2007; Tong et al., 2009; Eissa et al., 2016). Therefore, validation that candidate reference genes have stable expression is critical for the acquisition of accurate qPCR data and the experimental approaches that incorporate this technique.

To our knowledge, the only previous example of reference gene validation in *L. stagnalis* is for *EF1α* in the CNS (Foster, Lukowiak & Henry, 2015). Other experiments have used reference genes without validation, including *EF1α* (Gust et al., 2013b; Shimizu et al., 2013), *GAPDH* (Aleksic & Feng, 2012), *ACTB* (Senatore & Spafford, 2012; Hatakeyama et al., 2013; Carter et
al., 2015) and TUBB (Bavan et al., 2012; Korneev et al., 2013; Gust et al., 2013b; Flynn et al., 2014; Carter et al., 2015; Benatti et al., 2017). To establish a more rigorous foundation for future research using RT-qPCR, we present an analysis of seven candidate reference genes across six tissues of interest (tentacles, lips, foot, penis, mantle, and CNS) in L. stagnalis.

We investigated the stability of Lst-EF1a, Lst-GAPDH, Lst-ACTB, Lst-TUBB and Lst-ubiquitin (UBI) as they are commonly employed reference genes. Genes encoding the prenylated rab acceptor protein 1 (Lst-Rapac1) and a voltage gated potassium channel (Lst-VGKC) were also assessed as analogs of these genes have recently been found to be stable in the terrestrial gastropod Cepaea nemoralis (Affenzeller, Cerveau & Jackson, 2018).

Methods

Care of Snails

Animal use was consistent with the Canadian Council for Animal Care guidelines. A colony of Lymnaea stagnalis was bred and maintained in the animal care facility at StFX. The animals were exposed to a photoperiod matched to natural daylight patterns in Antigonish, Nova Scotia. The water in the animals’ tanks was changed three times per week. The animals were fed fish food and romaine lettuce ad libitum plus sinking protein pellets once per week.

Total RNA Extraction

Total RNA was collected from a total of six biological replicates of L. stagnalis. To prepare for extraction of total RNA, animals were anesthetized in 0.125% 1-phenoxy-2-propanol dissolved in Lymnaea saline for up to 30 minutes (Wyeth et al., 2009). The animals were
dissected to remove the shell and the digestive tract. Six tissues were isolated: CNS, tentacles, lips, penis, foot and mantle. The tissues of each type from two animals were combined and then flash frozen in liquid nitrogen, shredded with razor blades and weighed to ensure a mass between 10–50 mg. Dissected tissues were added to 500 µL of TRIzol reagent (ThermoFisher, Waltham, MA). Tissue solutions were thoroughly homogenized with a PowerGen 125 tissue homogenizer (ThermoFisher, Waltham, MA). Total RNA was extracted from the homogenized tissue via addition of 0.5 volumes of a 24:1 solution of chloroform and isoamyl alcohol. The aqueous layer was separated, and RNA was precipitated with isopropyl alcohol. RNA precipitate was transferred to the spin column of the E.Z.N.A.® Mollusc RNA Kit (Omega Bio-Tek, Norcross, GA) where the RNA was washed and subjected to the on-column DNase I treatment as per the manufacturer’s instructions. To confirm the effectiveness of the DNase I treatment, PCR was performed with primer sets for *Lst-tyrosine hydroxylase* and *Lst-EF1α* with total RNA samples as a template. If no amplification was observed after 40 cycles, the RNA samples were judged to be free of gDNA contamination.

Total RNA was quantified in a QuBit 3.0 (ThermoFisher, Waltham, MA). Yields of 50–200 ng/µL were common for roughly 40 mg of tissue mass. A sample of total RNA was measured with a spectrophotometer to confirm a 260:280 ratio of 1.8–2.0. Lastly, 5 µL of each RNA sample was denatured in 5 µL of 2X RNA Loading Dye (New England Biolabs, Ipswich, MA) at 75°C for 10 minutes and then immediately placed on ice. The RNA samples were loaded on a 1% agarose non-denaturing gel and run alongside a single stranded RNA ladder (New England Biolabs) to verify that the 28S and 18S rRNA bands were intact and the RNA samples were not degraded.
**Reverse Transcription PCR**

A library of cDNA was prepared from total RNA from each *L. stagnalis* tissue. RT-PCR was performed with iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, Hercules, CA) which contains a mixture of oligo(dT) primers and random hexamers. For RT-PCR reactions, 20 ng of RNA was added to 4 µL of iScript Supermix and topped up to 20 µL with molecular grade water as recommended by the manufacturer. RT-PCR took place in a Bio-Rad CFX Connect thermocycler (Bio-Rad Laboratories; Hercules, CA). The RT-PCR program consisted of 25°C for 5 minutes, 46°C for 20 minutes and 95°C for 1 minute.

**Design, validation and optimization of primers for quantitative PCR**

Candidate primers intended for qPCR were designed with CLC Main Workbench software (Qiagen, Hilden, DE). Primers were designed to have a length of 19-23 bp, a melting temperature between 57-62°C, a GC content between 40-60% and produce a product no more than 200 bp long. Additionally, the GC content of each primer was kept within 10% of its counterpart, and the melting temperature of each was kept within 3°C of its counterpart. Two to six sets of primers were designed for each gene so that optimal primers could be selected for the qPCR reactions. The primers were tested on combined cDNA samples to minimize tissue-specific bias. Melt curves were performed to verify that one product was amplified. If the primers produced a single product, then 5 µL of the PCR reactions were run on a 1% (w/v) agarose gel at 60 volts for roughly 30 minutes alongside a 50 bp DNA Ladder (New England BioLabs). The agarose gels were analyzed with a Bio-Rad ChemiDoc (Bio-Rad Laboratories) to visualize the size of the product (Figure S5). Ultimately, one set of primers was selected for each
candidate reference gene based on the quality of the PCR product. The primers for each
candidate reference gene are listed in Table 1.

After the optimal primer sets were selected based on reaction specificity, primer efficiencies
were calculated for each primer set. Primer efficiency curves were generated from RT-qPCR
reactions on serial dilutions of RNA. The first reaction contained 100 ng total RNA and four
more subsequent 1:5 dilutions were performed to generate five Cq values from serial dilutions.

Quantitative PCR

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE)
guidelines were followed throughout the collection of qPCR data (Bustin et al., 2009). Two
different methods were used to detect cDNA amplification. For Lst-EF1α, a hydrolysis probe
reaction was used. The hydrolysis probe is a gene-specific fluorescent marker that only
generates fluorescence as Lst-EF1α amplifies. The reaction mixture included 2 µL of cDNA with
10 µL of Bio-Rad SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories, USA), 600
nM primer concentration, 250 nM probe concentration and topped with H2O treated with diethyl
pyrocarbonate (DEPC) to produce a 20 µL reaction. Three technical replicates were performed
for each of the six biological replicates.

Amplification of all other genes was detected with SyBR Green dye. SyBR Green generates
fluorescence based on the synthesis of double-stranded DNA. The reactions contained 2 µL of
cDNA with 10 µL of Bio-Rad SsoAdvanced Universal SyBR Mix, 600 nM forward and reverse
primer concentration, and topped to 20 µL with DEPC H2O. Three technical replicates were
performed for each of the six biological replicates. The qPCR reactions took place in a Bio-Rad
CFX Connect thermocycler running a custom program. The custom qPCR program consisted of
95°C for 30s; 40 cycles of 95°C for 15s, 55°C for 30s. The plate was read by the machine to
measure fluorescence at the end of each cycle.

Results
Primer specificity and efficiency
Primers for all candidate reference genes were evaluated to ensure that they could produce
consistent results and not amplify off-target products or generate primer dimers. EF1α was
measured with a specific molecular probe whereas other genes were measured with SYBR green
fluorescent dye. Each primer pair generated single peaks in melting curves after qPCR and single
bands at the correct size after electrophoresis in 1.5% agarose gels. Additionally, no
amplification was observed in controls that lacked reverse transcriptase in the RT-PCR or lacked
cDNA template in qPCR. Thus, primer pairs specifically amplified a single cDNA target. Based
on the standard curves, primer set efficiencies ranged from 90% (GAPDH and Rapac1) to 106%
(ACTB) with correlation coefficients (R²) of >0.980 (Table 1).

Expression levels of reference genes
The reference genes displayed a wide range expression levels when the data was pooled across
all tissues. Based on Cq values (the point at which the fluorescence of the reaction is detectable
as a true signal), these results showed that Lst-UBI was the most highly expressed gene 20.03 ±
whereas Lst-VGKC was the most lowly expressed gene 30.20 ± 0.36 (Table 2). Lst-UBI also had the smallest overall Cq range whereas Lst-VGKC was the most variably expressed gene overall as measured by the standard error of the mean (Figure 1). The remaining genes had relatively moderate levels of expression with similar ranges of Cq values.

**Expression stability**

The expression stability of each gene was assessed with four computational algorithms: geNorm (Vandesompele et al., 2002), NormFinder (Andersen, Jensen & Ørntoft, 2004), BestKeeper (Pfaffl et al., 2004), and RefFinder (Xie et al., 2012). GeNorm ranks candidates by their expression stability (M) values that are assigned following pair-wise variation measurements among genes. Genes with an M-value of M<1.5 are considered to be stable. The NormFinder algorithm compares intra-group variation (i.e. mRNA levels of one gene within the tissue of interest) to inter-group variation (i.e. mRNA levels of other genes in the same tissues) and assigns genes a stability value based on variation among Cq values. BestKeeper judges reference gene stability based on the standard deviation (SD) for each gene, thus, genes that have a smaller SD will be ranked more highly by BestKeeper. Finally, RefFinder was used to combine the ranked results from each algorithm and assign each gene an overall rank. All four algorithms used for assessing expression stability were highly congruent in their ranking of candidate reference genes based on the pooled data from all tissue samples (Figure 2). GeNorm and NormFinder rated Lst-GAPDH and Lst-EF1α as the top two most stable reference genes whereas
BestKeeper ranked *Lst-Rapac1* as the most stable and *Lst-GAPDH* as the second most stable.

Overall, RefFinder ranked *Lst-GAPDH* and *Lst-EF1α* as the two strongest candidates.

**Tissue-specific expression profiles and stability of reference genes**

The candidate reference genes showed a variety of expression levels across tissues (Figure S1).

*Lst-UBI* was the most highly expressed gene in every tissue as it produced the smallest mean Cq values, and also displayed the smallest Cq ranges for most tissues. Conversely, *Lst-VGKC* was the lowliest expressed gene in every tissue and displayed large Cq ranges. Generally, most genes were highly and consistently expressed in the CNS and lips, whereas expression was generally lower and less consistent in the foot and mantle. *Lst-ACTB* and *Lst-TUBB* exhibited especially large discrepancies in expression levels between the foot, mantle, and the other tissues. In contrast, the foot and mantle expressed *Lst-Rapac1* and *Lst-UBI* at similar levels compared to the other tissues.

Similar to the overall analysis, all algorithms were highly congruent in their tissue-specific ranking of candidate reference genes, and thus only the results from the RefFinder analysis (which combines the rankings from the other three algorithms) are presented in Figure 3 (tissue-specific results for other algorithms are reported in Figures S2, S3, S4). *Lst-GAPDH* and *Lst-EF1α* were the highest ranked candidate reference genes in the CNS, tentacles, lips, and mantle. *Lst-GAPDH* and *Lst-TUBB* were the top two most stable genes in the penis. *Lst-EF1α* and *Lst-ACTB* were the most highly ranked in the foot. Overall, *Lst-GAPDH* and *Lst-EF1α* were the strongest and most consistent candidate reference gene across most tissues (Figure 4). It is also worth noting that according to geNorm, *Lst-EF1α* was the only gene to meet its stability
threshold of $M < 1.50$ in every tissue whereas $Lst$-$GAPDH$ met the threshold in every tissue except the mantle which had a stability value of 1.56.

Discussion

We assessed seven candidate reference genes in six tissues of interest (CNS, tentacles, lips, penis, foot, mantle) as well as the pooled data from all tissues. There were consistent trends among the genes and the rankings produced by the different algorithms were generally congruent. Overall, we found $Lst$-$GAPDH$ and $Lst$-$EF1\alpha$ to be the most stable pair of reference genes to use in whole-body and cross-tissue comparisons of gene expression. These genes likely performed exceptionally well with geNorm as they display similar expression patterns among tissues. The tissue-specific analyses demonstrated some discrepancies between the algorithms compared to the pooled analysis. $Lst$-$GAPDH$ and $Lst$-$EF1\alpha$ were highly ranked in most tissues, but $Lst$-$VGKC$ was an unsuitable reference genes for every tissue.

In the context of previous research on RT-qPCR reference gene stability in molluscs, $GAPDH$ and $EF1\alpha$ appear to be the most favorable reference genes overall (Table 3). In one study of $L. stagnalis$, $EF1\alpha$ was identified to be stable in the CNS under heat stress (Foster, Lukowiak & Henry, 2015). $EF1\alpha$ has also been identified to be highly stable in several mollusc species and thus is a strong candidate reference gene across the phylum (Wan et al., 2011; Cubero-Leon et al., 2012; Moreira et al., 2014; García-Fernández et al., 2016; Huan, Wang & Liu, 2016). An analysis of reference genes in the freshwater snail $Bellamya aeruginosa$ showed that $EF1\alpha$ and $GAPDH$ were stable in the tentacles and penis but more variable in the foot (Liu et al., 2015), similar to our findings (albeit with some discrepancies of rankings in the penis and foot tissues).
GAPDH has also been shown to be a stable reference gene in some molluscs (Huan, Wang & Liu, 2016; Martínez-Escauriaza et al., 2018) but was reported as unsuitable in others (Wan et al., 2011; García-Fernández et al., 2016). Additionally, GAPDH is highly stable in the terrestrial gastropod *Cepaea nemoralis*, but expression levels are subject to great variation between seasons (Affenzeller, Cerveau & Jackson, 2018). Lst-UBI was ranked poorly overall by all of the algorithms we employed. However, Lst-UBI displayed a relatively consistent expression profile and has shown promise in other species (Sirakov et al., 2009; García-Fernández et al., 2016; Affenzeller, Cerveau & Jackson, 2018). It is likely that the high level of Lst-UBI expression in all tissues makes it suboptimal reference gene (see Figure 1 and Supplementary Figure 1). Alpha-tubulin (*TUBA*) has generally produced positive results as a reference gene in several molluscan species (Sirakov et al., 2009; Cubero-Leon et al., 2012; Moreira et al., 2014). However, *TUBA* was unstable in *C. nemoralis* (Affenzeller, Cerveau & Jackson, 2018) and we also found Lst-*TUBB* to be generally unstable. *ACTB* has generally produced negative results as a candidate reference gene in molluscs (Cubero-Leon et al., 2012; Moreira et al., 2014; Liu et al., 2015; García-Fernández et al., 2016; Huan, Wang & Liu, 2016) and the results here from *L. stagnalis* are congruent with these previous findings. Finally, *Rapac1* and *VGKC* were identified as strong novel reference genes in *C. nemoralis* (Affenzeller, Cerveau & Jackson, 2018). Lst-*Rapac1* did show relative stability in the foot and mantle but Lst-*VGKC* was highly unstable in all tissues examined. Such differences may represent lineage specific idiosyncrasies between these species as these two genes were identified *de novo* from a transcriptome analysis of different tissues in *C. nemoralis* (Affenzeller, Cerveau & Jackson, 2018).
Lst-GAPDH and Lst-EF1α were constitutively and stably expressed across all of the tissue types we investigated which confirms their use as reference genes in other species (Reid et al., 2006; Scharlaken et al., 2008; Morga et al., 2010; Mauriz et al., 2012). However, it would be prudent to verify their stability prior to their use as reference genes under experimental conditions that vary from those employed here such as after exposure to pharmaceutical compounds, comparisons across seasons or physiological conditions such as reproductive state, starvation or hibernation (García-Fernández et al., 2016; Martínez-Escauriaza et al., 2018; Affenzeller, Cerveau & Jackson, 2018).

Insert Table 3 here

Conclusion

The validation of stable reference genes is important to the acquisition of reliable gene expression data. Therefore, it is important to perform a species-specific verification of reference gene stability before undertaking RT-qPCR experiments. The variable expression of the seven genes among the six tissue types we investigated demonstrates that it is critical to select reference genes based on the tissues of interest. Lst-UBI and Lst-VGKC were poorly ranked whereas Lst-ACTB, Lst-TUBB and Lst-Rapac1 varied among tissues. Lst-GAPDH and Lst-EF1α were the most stable genes overall among tissues as well as in our pooled analyses and therefore should be considered for future RT-qPCR experiments with L. stagnalis.

Acknowledgements
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Figure 1

Cq values for each candidate reference gene from all tissues.

Box plot: upper and lower box limits indicate 25th and 75th percentiles, dark/light grey interface indicates the median, ‘x’ indicates the mean, and whiskers indicate the minimum/maximum values. Note that low Cq values equate to high expression levels. Data was collected from six biological replicates with three technical replicates for each.
Figure 2

Comprehensive rankings assigned to each candidate reference gene.

Rankings were based on pooled Cq values from all tissues and assigned by geNorm, NormFinder, BestKeeper and RefFinder. *Indicates that both genes were equally recommended.
Figure 3

RefFinder comprehensive rankings for all candidate reference genes by tissue.

RefFinder calculates rankings as the geometric mean of the rankings assigned by geNorm, NormFinder and BestKeeper. Genes are ranked in order from the least stable to the most stable in each panel.
Reffinder comprehensive rankings for all candidate reference genes colour coded by performance.

Genes are colour coded based on stability with cyan representing the highest stability and magenta indicating the lowest while grey represents the mid-range.
Table 1 (on next page)

Description of all primers used to amplify candidate reference genes in *Lymnaea stagnalis*.

Primers labeled “For” are forward primers and primers labeled “Rev” are reverse primers, all sequences are written in the 5’ to 3’ direction. “Probe” represents the sequence of the hydrolysis probe used with the EF1α primer set. The amplification efficiency was determined from each reference gene primer set following RT-qPCR with five 1:5 serial dilutions of total RNA from 100 ng.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’ to 3’)</th>
<th>Function</th>
<th>Product Size (bp)</th>
<th>Tm (˚C)</th>
<th>Efficiency</th>
<th>r²</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lst-ACTB</td>
<td>For [AGGCCAACAGAGAAAGA]</td>
<td>Cell structure and motility</td>
<td>97</td>
<td>56</td>
<td>2.12</td>
<td>0.999</td>
<td>KX387883</td>
</tr>
<tr>
<td>Lst-EF1α</td>
<td>For [ACCACAACGTGCACTTGT]</td>
<td>Delivery of tRNA for protein synthesis</td>
<td>85</td>
<td>59</td>
<td>2.00</td>
<td>0.998</td>
<td>MH687364</td>
</tr>
<tr>
<td>Lst-GAPDH</td>
<td>For [CAACAACCGACAAAGCAA]</td>
<td>Carbohydrate metabolism</td>
<td>93</td>
<td>55</td>
<td>1.82</td>
<td>0.988</td>
<td>MH687363</td>
</tr>
<tr>
<td>Lst-Rapac</td>
<td>For [GGCTCTTTTCCCTTGGGGA]</td>
<td>Cellular trafficking</td>
<td>124</td>
<td>58</td>
<td>1.82</td>
<td>0.989</td>
<td>MH687365</td>
</tr>
<tr>
<td>Lst-TUBB</td>
<td>For [GGCTAGGGGTAGAAAATG]</td>
<td>Microtubule element – cell structure</td>
<td>130</td>
<td>56</td>
<td>1.80</td>
<td>0.993</td>
<td>KX387887</td>
</tr>
<tr>
<td>Lst-UBI</td>
<td>For [GTATTTGTGTGGCTGTTGTTT]</td>
<td>Regulate protein function</td>
<td>105</td>
<td>59</td>
<td>1.94</td>
<td>0.993</td>
<td>MH687367</td>
</tr>
<tr>
<td>Lst-VGKC</td>
<td>For [TGCCCTCTTTGCTGCTTGT]</td>
<td>Maintenance of cell membrane potential</td>
<td>99</td>
<td>60</td>
<td>1.82</td>
<td>0.997</td>
<td>MH687366</td>
</tr>
</tbody>
</table>

Primers labeled “For” are forward primers and primers labeled “Rev” are reverse primers, all sequences are written in the 5’ to 3’ direction. “Probe” represents the sequence of the hydrolysis probe used with the EF1α primer set. The amplification efficiency was determined from each reference gene primer set following RT-qPCR with five 1:5 serial dilutions of total RNA from 100 ng.
Mean Cq values and standard errors of the mean (SEM) of reference genes for tissues in *Lymnaea stagnalis*.

Means were calculated from six biological replicates, all with three technical replicates each.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Lst-ACTB Cq Mean</th>
<th>SEM</th>
<th>Lst-EF1α Cq Mean</th>
<th>SEM</th>
<th>Lst-GAPDH Cq Mean</th>
<th>SEM</th>
<th>Lst-Rapac1 Cq Mean</th>
<th>SEM</th>
<th>Lst-TUBB Cq Mean</th>
<th>SEM</th>
<th>Lst-UBI Cq Mean</th>
<th>SEM</th>
<th>Lst-VGKC Cq Mean</th>
<th>SEM</th>
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<td>CNS</td>
<td>22.04</td>
<td>0.23</td>
<td>23.85</td>
<td>0.21</td>
<td>24.07</td>
<td>0.16</td>
<td>26.27</td>
<td>0.13</td>
<td>21.48</td>
<td>0.22</td>
<td>19.75</td>
<td>0.12</td>
<td>29.32</td>
<td>0.43</td>
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<td>Tentacle</td>
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<td>0.42</td>
<td>24.93</td>
<td>0.50</td>
<td>25.17</td>
<td>0.38</td>
<td>27.40</td>
<td>0.22</td>
<td>22.47</td>
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<td>20.47</td>
<td>0.23</td>
<td>30.10</td>
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<td>Lips</td>
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<td>0.35</td>
<td>23.89</td>
<td>0.19</td>
<td>24.06</td>
<td>0.17</td>
<td>25.96</td>
<td>0.21</td>
<td>21.62</td>
<td>0.20</td>
<td>19.23</td>
<td>0.22</td>
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<tr>
<td>Penis</td>
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<td>0.32</td>
<td>25.42</td>
<td>0.46</td>
<td>24.72</td>
<td>0.36</td>
<td>27.42</td>
<td>0.26</td>
<td>23.01</td>
<td>0.29</td>
<td>20.52</td>
<td>0.16</td>
<td>31.43</td>
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<tr>
<td>Foot</td>
<td>24.78</td>
<td>0.85</td>
<td>27.43</td>
<td>0.67</td>
<td>26.62</td>
<td>0.82</td>
<td>27.00</td>
<td>0.29</td>
<td>26.31</td>
<td>0.89</td>
<td>20.41</td>
<td>0.22</td>
<td>29.72</td>
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<tr>
<td>Mantle</td>
<td>25.40</td>
<td>0.89</td>
<td>27.20</td>
<td>0.55</td>
<td>25.75</td>
<td>0.39</td>
<td>27.12</td>
<td>0.39</td>
<td>24.56</td>
<td>0.70</td>
<td>19.82</td>
<td>0.22</td>
<td>30.42</td>
<td>0.60</td>
</tr>
</tbody>
</table>

1. Table 1. Mean Cq values and standard errors of the mean (SEM) of reference genes for tissues in *Lymnaea stagnalis*.

2. Means were calculated from six biological replicates, all with three technical replicates each.
Table 3 (on next page)

Summary of the stability rankings of reference genes from studies conducted in molluscs.

ARF1, adp-ribosylation factor 1; BGLU, beta-glucuronidase; COX1, cytochrome c oxidase subunit 1; CY, cyclophilin; CYP4, cytochrome p450 family 4; DNARP, DNA repair protein; DRP2, DNA-directed RNA polymerase II; FIB3, fibronectin type III domain containing protein; GTP8, GTP-binding protein; H2A, histone H2A; HEL, RNA helicase; HNRPQ, heterogeneous nuclear ribonucleoprotein q; HPRT, hypoxanthine phosphoribosyltransferase 1; NAD4, NADH dehydrogenase subunit 4; RNAP, RNA-directed DNA polymerase; RPL5, ribosomal protein L5; RPL5, ribosomal protein L7; RPS4, 40S ribosomal protein S4; RPS27, 40S ribosomal protein S27; SDHA, succinate dehydrogenase; UBC, ubiquitin-conjugating enzyme.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Most stable gene(s)</th>
<th>Least stable gene(s)</th>
<th>Other Genes Tested</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Gastropods</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Haliotis discus</em></td>
<td>EF1α/RPL5</td>
<td>18S rRNA</td>
<td>ACTB, BGLU, CY, GAPDH, H2A, HPRT, SDHA, TUBB, UBC, CYP4</td>
<td>Wan et al. 2011</td>
</tr>
<tr>
<td><em>Bellamya aeruginosa</em></td>
<td>RPL7</td>
<td>ACTB</td>
<td>18S rRNA, EF1α, GAPDH, TUBB, H2A, DRP2</td>
<td>Liu et al. 2016</td>
</tr>
<tr>
<td><em>Cepaea nemoralis</em></td>
<td>EF1α/ACTB</td>
<td>GAPDH</td>
<td>DNARP, FIB3, GTP8, Rapac1, RNAP, TUBA, UBI, VGKC</td>
<td>Affenzeller et al. 2018</td>
</tr>
<tr>
<td><em>Lymnaea stagnalis</em></td>
<td>EF1α/GAPDH</td>
<td>VGKC</td>
<td>ACTB, TUBB, UBI, Rapac1</td>
<td>Present study</td>
</tr>
<tr>
<td><strong>Cephalopods</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>Octopus vulgaris</em></td>
<td>TUBA/UBI</td>
<td>18S rRNA</td>
<td>16S rRNA, ACTB, EF1α, TUBA</td>
<td>Sirakov et al. 2009</td>
</tr>
<tr>
<td>Species</td>
<td>Reference Genes</td>
<td>Assayed Genes</td>
<td>References</td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>-----------------------</td>
<td>---------------------</td>
<td>---------------------</td>
<td></td>
</tr>
<tr>
<td><em>Octopus vulgaris</em></td>
<td>UBI</td>
<td>ACTB</td>
<td>18S rRNA, EF1α, GAPDH, TUBA</td>
<td>García-Fernández et al. 2016</td>
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<tr>
<td><strong>Bivalves</strong></td>
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</tr>
<tr>
<td><em>Ostrea edulis</em></td>
<td>EF1α/GAPDH</td>
<td>ACTB</td>
<td>UBI, RPL5</td>
<td>Morga et al. 2010</td>
</tr>
<tr>
<td><strong>Mytilus edulis</strong></td>
<td>EF1α/18S rRNA</td>
<td>ACTB</td>
<td>28S rRNA, TUBA, HEL</td>
<td>Cubero-Leon et al. 2012</td>
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<tr>
<td><em>Mytilus galloprovincialis</em></td>
<td>EF1α</td>
<td>ACTB</td>
<td>18S rRNA, TUBA</td>
<td>Moreira et al. 2015</td>
</tr>
<tr>
<td><em>Ruditapes philippinarum</em></td>
<td>TUBA</td>
<td>ACTB</td>
<td>18S rRNA, EF1α</td>
<td>Moreira et al. 2015</td>
</tr>
<tr>
<td><strong>Crassostrea gigas</strong></td>
<td>EF1α</td>
<td>RPS18</td>
<td>ACTB, ARF1, GAPDH, HNRPQ, UBC</td>
<td>Huan et al. 2016</td>
</tr>
<tr>
<td>Mytilus galloprovincialis</td>
<td>GAPDH/RPS4</td>
<td>NAD4/18S rRNA</td>
<td>ACTB, COX1, GAPDH, RPS27, TIF5A</td>
<td>Martínez-Escauriaza et al. 2018</td>
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<tr>
<td>--------------------------</td>
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<tr>
<td>ARF1, adp-ribosylation factor 1; BGLU, beta-glucuronidase; COX1, cytochrome c oxidase subunit 1; CY, cyclophilin; CYP4, cytochrome p450 family 4; DNARP, DNA repair protein; DRP2, DNA-directed RNA polymerase II; FIB3, fibronectin type III domain containing protein; GTP8, GTP-binding protein; H2A, histone H2A; HEL, RNA helicase; HNRPQ, heterogeneous nuclear ribonucleoprotein q; HPRT, hypoxanthine phosphoribosyltransferase 1; NAD4, NADH dehydrogenase subunit 4; RNAP, RNA-directed DNA polymerase; RPL5, ribosomal protein L5; RPL5, ribosomal protein L7; RPS4, 40S ribosomal protein S4; RPS27, 40S ribosomal protein S27; SDHA, succinate dehydrogenase; UBC, ubiquitin-conjugating enzyme.</td>
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