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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-acti*n (*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.*

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2 ***Lymnaea stagnalis***

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15 **Abstract**

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

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38

39 **Background**

40 *Lymnaea stagnalis* is a freshwater snail with an extensive history of proximate mechanism
41 research. Previous studies have primarily focused on its neurobiology, as well as its
42 endocrinology, immunology and shell morphology (Chase, 2002; Benjamin, 2008). The central
43 nervous system (CNS) is relatively simple, with approximately 20,000 neurons, many of which
44 are large and easily identifiable, making them amenable for studies of learning, memory, motor
45 pattern generation, neuronal regeneration and synapse formation (Lukowiak; Elliott & Susswein,
46 2002; Chase, 2002; Lukowiak et al., 2003; Getz et al., 2018). Parallel investigations into the
47 neuroendocrine (Koene, 2010; Pirger et al., 2010) and immune (van der Knaap, Adema &
48 Sminia, 1993; Gust et al., 2013a) systems of *L. stagnalis* and other gastropods have improved
49 our understanding of reproductive behaviour, immunity and toxicology of *L. stagnalis*.
50 Additionally, *L. stagnalis* has been used as a research organism to study the molecular
51 mechanisms that guide shell formation (Boer & Witteveen, 1980; Ebanks, O'Donnell & Grosell,
52 2010; Hohagen & Jackson, 2013; Jackson, Herlitze & Hohagen, 2016; Herlitze et al., 2018).

53

54 A broad range of traditional techniques have been used in past studies, but modern molecular
55 genetics have yet to be thoroughly incorporated into the study of *L. stagnalis.* Methods to study
56 gene expression such as reverse transcription quantitative PCR (RT-qPCR) and *in situ*
57 hybridization (among others) have been established but have not yet been used extensively. The
58 few studies using RT-qPCR have spanned several topics including chirality, learning, and
59 memory (Foster, Lukowiak & Henry, 2015; Davison et al., 2016; Korneev et al., 2018; Dong et
60 al., 2018). Additionally, a handful of studies have used *in situ* hybridization in this species,

61 primarily in sections and whole mounts of the CNS, as well as larvae (Dirks et al., 1989; Boer et
62 al., 1992; Croll & Minnen, 1992; Sadamoto et al., 2004). Our goal here is to continue to build the
63 foundation for further molecular genetics studies in *L. stagnalis*, by validating candidate
64 reference genes for RT-qPCR.
65
66 RT-qPCR is a robust technique for the quantification of the mRNA for a specific transcript. It
67 can be used alongside *in situ* hybridization or other visualization techniques to establish patterns
68 of gene expression in animals. For such experiments, relative quantification against one or more
69 reference genes is the most common choice to compare gene expression across samples
70 (Schmittgen & Livak, 2008; Ruijter et al., 2009). Historically, highly expressed cellular
71 maintenance genes have been haphazardly selected as reference genes for qPCR experiments in
72 many species because expression of such genes was thought to be inherently stable (Kozera &
73 Rapacz, 2013). However, many of these genes have been shown to have unstable expression in
74 several species of plants and animals, highlighting the importance of species-specific validation
75 prior to use (Dheda et al., 2004; Barber et al., 2005; Jonge et al., 2007; Tong et al., 2009; Eissa et
76 al., 2016). Therefore, validation that candidate reference genes have stable expression is critical
77 for the acquisition of accurate qPCR data and the experimental approaches that incorporate this
78 technique.
79
80 To our knowledge, the only previous example of reference gene validation in *L. stagnalis* is for
81 *EF1α* in the CNS (Foster, Lukowiak & Henry, 2015). Other experiments have used reference
82 genes without validation, including *EF1α* (Gust et al., 2013b; Shimizu et al., 2013), *GAPDH*
83 (Aleksic & Feng, 2012), *ACTB* (Senatore & Spafford, 2012; Hatakeyama et al., 2013; Carter et

84 al., 2015) and *TUBB* (Bavan et al., 2012; Korneev et al., 2013; Gust et al., 2013b; Flynn et al.,
85 2014; Carter et al., 2015; Benatti et al., 2017). To establish a more rigorous foundation for future
86 research using RT-qPCR, we present an analysis of seven candidate reference genes across six
87 tissues of interest (tentacles, lips, foot, penis, mantle, and CNS) in *L. stagnalis*.
88
89 We investigated the stability of *Lst-EF1α*, *Lst-GAPDH*, *Lst-ACTB*, *Lst-TUBB* and *Lst-ubiquitin*
90 (*UBI*) as they are commonly employed reference genes. Genes encoding the prenylated rab
91 acceptor protein 1 (*Lst-Rapac1*) and a voltage gated potassium channel (*Lst-VGKC*) were also
92 assessed as analogs of these genes have recently been found to be stable in the terrestrial
93 gastropod *Cepaea nemoralis* (Affenzeller, Cerveau & Jackson, 2018).
94

95 # Methods

96 ## Care of Snails

97 Animal use was consistent with the Canadian Council for Animal Care guidelines. A colony of
98 *Lymnaea stagnalis* was bred and maintained in the animal care facility at StFX. The animals
99 were exposed to a photoperiod matched to natural daylight patterns in Antigonish, Nova Scotia.
100 The water in the animals' tanks was changed three times per week. The animals were fed fish
101 food and romaine lettuce *ad libitum* plus sinking protein pellets once per week.
102

103 ## Total RNA Extraction

104 Total RNA was collected from a total of six biological replicates of *L. stagnalis*. To prepare for
105 extraction of total RNA, animals were anesthetized in in 0.125% 1-phenoxy-2-propanol
106 dissolved in *Lymnaea* saline for up to 30 minutes (Wyeth et al., 2009). The animals were

107 dissected to remove the shell and the digestive tract. Six tissues were isolated: CNS, tentacles,
108 lips, penis, foot and mantle. The tissues of each type from two animals were combined and then
109 flash frozen in liquid nitrogen, shredded with razor blades and weighed to ensure a mass between
110 10–50 mg. Dissected tissues were added to 500 μL of TRIzol reagent (ThermoFisher, Waltham,
111 MA). Tissue solutions were thoroughly homogenized with a PowerGen 125 tissue homogenizer
112 (ThermoFisher, Waltham, MA). Total RNA was extracted from the homogenized tissue via
113 addition of 0.5 volumes of a 24:1 solution of chloroform and isoamyl alcohol. The aqueous layer
114 was separated, and RNA was precipitated with isopropyl alcohol. RNA precipitate was
115 transferred to the spin column of the E.Z.N.A.® Mollusc RNA Kit (Omega Bio-Tek, Norcross,
116 GA) where the RNA was washed and subjected to the on-column DNAse I treatment as per the
117 manufacturer's instructions. To confirm the effectiveness of the DNase I treatment, PCR was
118 performed with primer sets for *Lst-tyrosine hydroxylase* and *Lst-EF1α* with total RNA samples
119 as a template. If no amplification was observed after 40 cycles, the RNA samples were judged to
120 be free of gDNA contamination.
121
122 Total RNA was quantified in a QuBit 3.0 (ThermoFisher, Waltham, MA). Yields of 50–200
123 ng/μL were common for roughly 40 mg of tissue mass. A sample of total RNA was measured
124 with a spectrophotometer to confirm a 260:280 ratio of 1.8–2.0. Lastly, 5 μL of each RNA
125 sample was denatured in 5 μL of 2X RNA Loading Dye (New England Biolabs, Ipswich, MA) at
126 75°C for 10 minutes and then immediately placed on ice. The RNA samples were loaded on a
127 1% agarose non-denaturing gel and run alongside a single stranded RNA ladder (New England
128 Biolabs) to verify that the 28S and 18S rRNA bands were intact and the RNA samples were not
129 degraded.

130

131 **Reverse Transcription PCR**

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

139

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

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

153 candidate reference gene based on the quality of the PCR product. The primers for each
154 candidate reference gene are listed in Table 1.
155
156 After the optimal primer sets were selected based on reaction specificity, primer efficiencies
157 were calculated for each primer set. Primer efficiency curves were generated from RT-qPCR
158 reactions on serial dilutions of RNA. The first reaction contained 100 ng total RNA and four
159 more subsequent 1:5 dilutions were performed to generate five Cq values from serial dilutions.
160
161 **Quantitative PCR**
162 The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE)
163 guidelines were followed throughout the collection of qPCR data (Bustin et al., 2009). Two
164 different methods were used to detect cDNA amplification. For *Lst-EF1α*, a hydrolysis probe
165 reaction was used. The hydrolysis probe is a gene-specific fluorescent marker that only
166 generates fluorescence as *Lst-EF1α* amplifies. The reaction mixture included 2 μL of cDNA with
167 10 μL of Bio-Rad SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories, USA), 600
168 nM primer concentration, 250 nM probe concentration and topped with H_2O treated with diethyl
169 pyrocarbonate (DEPC) to produce a 20 μL reaction. Three technical replicates were performed
170 for each of the six biological replicates.
171
172 Amplification of all other genes was detected with SyBR Green dye. SyBR Green generates
173 fluorescence based on the synthesis of double-stranded DNA. The reactions contained 2 μL of
174 cDNA with 10 μL of Bio-Rad SsoAdvanced Universal SyBR Mix, 600 nM forward and reverse
175 primer concentration, and topped to 20 μL with DEPC H_2O. Three technical replicates were

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176 performed for each of the six biological replicates. The qPCR reactions took place in a Bio-Rad
177 CFX Connect thermocycler running a custom program. The custom qPCR program consisted of
178 95°C for 30s; 40 cycles of 95°C for 15s, 55°C for 30s. The plate was read by the machine to
179 measure fluorescence at the end of each cycle.
180

181 Results

182 Primer specificity and efficiency

183 Primers for all candidate reference genes were evaluated to ensure that they could produce
184 consistent results and not amplify off-target products or generate primer dimers. *EF1α* was
185 measured with a specific molecular probe whereas other genes were measured with SYBR green
186 fluorescent dye. Each primer pair generated single peaks in melting curves after qPCR and single
187 bands at the correct size after electrophoresis in 1.5% agarose gels. Additionally, no
188 amplification was observed in controls that lacked reverse transcriptase in the RT-PCR or lacked
189 cDNA template in qPCR. Thus, primer pairs specifically amplified a single cDNA target. Based
190 on the standard curves, primer set efficiencies ranged from 90% (*GAPDH* and *Rapac1*) to 106%
191 (*ACTB*) with correlation coefficients (R^2) of >0.980 (Table 1).
192

193 **Insert Table 1 here**

194

195 Expression levels of reference genes

196 The reference genes displayed a wide range expression levels when the data was pooled across
197 all tissues. Based on Cq values (the point at which the fluorescence of the reaction is detectable
198 as a true signal), these results showed that *Lst-UBI* was the most highly expressed gene 20.03 ±

199 0.16 whereas *Lst-VGKC* was the most lowly expressed gene 30.20 ± 0.36 (Table 2). *Lst-UBI* also
200 had the smallest overall Cq range whereas *Lst-VGKC* was the most variably expressed gene
201 overall as measured by the standard error of the mean (Figure 1). The remaining genes had
202 relatively moderate levels of expression with similar ranges of Cq values.
203
204 **Insert Table 2 here**
205
206 **Expression stability**
207 The expression stability of each gene was assessed with four computational algorithms: geNorm
208 (Vandesompele et al., 2002), NormFinder (Andersen, Jensen & Ørntoft, 2004), BestKeeper
209 (Pfaffl et al., 2004), and RefFinder (Xie et al., 2012). GeNorm ranks candidates by their
210 expression stability (M) values that are assigned following pair-wise variation measurements
211 among genes. Genes with an M-value of $M<1.5$ are considered to be stable. The NormFinder
212 algorithm compares intra-group variation (i.e. mRNA levels of one gene within the tissue of
213 interest) to inter-group variation (i.e. mRNA levels of other genes in the same tissues) and
214 assigns genes a stability value based on variation among Cq values. BestKeeper judges reference
215 gene stability based on the standard deviation (SD) for each gene, thus, genes that have a smaller
216 SD will be ranked more highly by BestKeeper. Finally, RefFinder was used to combine the
217 ranked results from each algorithm and assign each gene an overall rank. All four algorithms
218 used for assessing expression stability were highly congruent in their ranking of candidate
219 reference genes based on the pooled data from all tissue samples (Figure 2). GeNorm and
220 NormFinder rated *Lst-GAPDH* and *Lst-EF1α* as the top two most stable reference genes whereas

221 BestKeeper ranked *Lst-Rapac1* as the most stable and *Lst-GAPDH* as the second most stable.
222 Overall, RefFinder ranked *Lst-GAPDH* and *Lst-EF1α* as the two strongest candidates.
223
224 **Tissue-specific expression profiles and stability of reference genes**
225 The candidate reference genes showed a variety of expression levels across tissues (Figure S1).
226 *Lst-UBI* was the most highly expressed gene in every tissue as it produced the smallest mean Cq
227 values, and also displayed the smallest Cq ranges for most tissues. Conversely, *Lst-VGKC* was
228 the lowliest expressed gene in every tissue and displayed large Cq ranges. Generally, most genes
229 were highly and consistently expressed in the CNS and lips, whereas expression was generally
230 lower and less consistent in the foot and mantle. *Lst-ACTB* and *Lst-TUBB* exhibited especially
231 large discrepancies in expression levels between the foot, mantle, and the other tissues. In
232 contrast, the foot and mantle expressed *Lst-Rapac1* and *Lst-UBI* at similar levels compared to the
233 other tissues.
234
235 Similar to the overall analysis, all algorithms were highly congruent in their tissue-specific
236 ranking of candidate reference genes, and thus only the results from the RefFinder analysis
237 (which combines the rankings from the other three algorithms) are presented in Figure 3 (tissue-
238 specific results for other algorithms are reported in Figures S2, S3, S4). *Lst-GAPDH* and *Lst-*
239 *EF1α* were the highest ranked candidate reference genes in the CNS, tentacles, lips, and mantle.
240 *Lst-GAPDH* and *Lst-TUBB* were the top two most stable genes in the penis. *Lst-EF1α* and *Lst-*
241 *ACTB* were the most highly ranked in the foot. Overall, *Lst-GAPDH* and *Lst-EF1α* were the
242 strongest and most consistent candidate reference gene across most tissues (Figure 4). It is also
243 worth noting that according to geNorm, *Lst-EF1α* was the only gene to meet its stability

244 threshold of M<1.50 in every tissue whereas *Lst-GAPDH* met the threshold in every tissue
245 except the mantle which had a stability value of 1.56.
246

247 Discussion

248 We assessed seven candidate reference genes in six tissues of interest (CNS, tentacles, lips,
249 penis, foot, mantle) as well as the pooled data from all tissues. There were consistent trends
250 among the genes and the rankings produced by the different algorithms were generally
251 congruent. Overall, we found *Lst-GAPDH* and *Lst-EF1α* to be the most stable pair of reference
252 genes to use in whole-body and cross-tissue comparisons of gene expression. These genes likely
253 performed exceptionally well with geNorm as they display similar expression patterns among
254 tissues. The tissue-specific analyses demonstrated some discrepancies between the algorithms
255 compared to the pooled analysis. *Lst-GAPDH* and *Lst-EF1α* were highly ranked in most tissues,
256 but *Lst-VGKC* was an unsuitable reference genes for every tissue.
257

258 In the context of previous research on RT-qPCR reference gene stability in molluscs, *GAPDH*
259 and *EF1α* appear to be the most favorable reference genes overall (Table 3). In one study of *L.*
260 *stagnalis*, *EF1α* was identified to be stable in the CNS under heat stress (Foster, Lukowiak &
261 Henry, 2015). *EF1α* has also been identified to be highly stable in several mollusc species and
262 thus is a strong candidate reference gene across the phylum (Wan et al., 2011; Cubero-Leon et
263 al., 2012; Moreira et al., 2014; García-Fernández et al., 2016; Huan, Wang & Liu, 2016). An
264 analysis of reference genes in the freshwater snail *Bellamya aeruginosa* showed that *EF1α* and
265 *GAPDH* were stable in the tentacles and penis but more variable in the foot (Liu et al., 2015),
266 similar to our findings (albeit with some discrepancies of rankings in the penis and foot tissues).

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267 *GAPDH* has also been shown to be a stable reference gene in some molluscs (Huan, Wang &
268 Liu, 2016; Martínez-Escauriaza et al., 2018) but was reported as unsuitable in others (Wan et al.,
269 2011; García-Fernández et al., 2016). Additionally, *GAPDH* is highly stable in the terrestrial
270 gastropod *Cepaea nemoralis*, but expression levels are subject to great variation between seasons
271 (Affenzeller, Cerveau & Jackson, 2018). *Lst-UBI* was ranked poorly overall by all of the
272 algorithms we employed. However, *Lst-UBI* displayed a relatively consistent expression profile
273 and has shown promise in other species (Sirakov et al., 2009; García-Fernández et al., 2016;
274 Affenzeller, Cerveau & Jackson, 2018). It is likely that the high level of *Lst-UBI* expression in
275 all tissues makes it suboptimal reference gene (see Figure 1 and Supplementary Figure 1). *Alpha-*
276 *tubulin* (*TUBA*) has generally produced positive results as a reference gene in several molluscan
277 species (Sirakov et al., 2009; Cubero-Leon et al., 2012; Moreira et al., 2014). However, *TUBA*
278 was unstable in *C. nemoralis* (Affenzeller, Cerveau & Jackson, 2018) and we also found *Lst-*
279 *TUBB* to be generally unstable. *ACTB* has generally produced negative results as a candidate
280 reference gene in molluscs (Cubero-Leon et al., 2012; Moreira et al., 2014; Liu et al., 2015;
281 García-Fernández et al., 2016; Huan, Wang & Liu, 2016) and the results here from *L. stagnalis*
282 are congruent with these previous findings. Finally, *Rapac1* and *VGKC* were identified as strong
283 novel reference genes in *C. nemoralis* (Affenzeller, Cerveau & Jackson, 2018). *Lst-Rapac1* did
284 show relative stability in the foot and mantle but *Lst-VGKC* was highly unstable in all tissues
285 examined. Such differences may represent lineage specific idiosyncrasies between these species
286 as these two genes were identified *de novo* from a transcriptome analysis of different tissues in
287 *C. nemoralis* (Affenzeller, Cerveau & Jackson, 2018).
288

289 *Lst-GAPDH* and *Lst-EF1α* were constitutively and stably expressed across all of the tissue types
290 we investigated which confirms their use as reference genes in other species (Reid et al., 2006;
291 Scharlaken et al., 2008; Morga et al., 2010; Mauriz et al., 2012). However, it would be prudent to
292 verify their stability prior to their use as reference genes under experimental conditions that vary
293 from those employed here such as after exposure to pharmaceutical compounds, comparisons
294 across seasons or physiological conditions such as reproductive state, starvation or hibernation
295 (García-Fernández et al., 2016; Martínez-Escauriaza et al., 2018; Affenzeller, Cerveau &
296 Jackson, 2018).
297
298 **Insert Table 3 here**
299

300 Conclusion

301 The validation of stable reference genes is important to the acquisition of reliable gene
302 expression data. Therefore, it is important to perform a species-specific verification of reference
303 gene stability before undertaking RT-qPCR experiments. The variable expression of the seven
304 genes among the six tissue types we investigated demonstrates that it is critical to select
305 reference genes based on the tissues of interest. *Lst-UBI* and *Lst-VGKC* were poorly ranked
306 whereas *Lst-ACTB*, *Lst-TUBB* and *Lst-Rapac1* varied among tissues. *Lst-GAPDH* and *Lst-EF1α*
307 were the most stable genes overall among tissues as well as in our pooled analyses and therefore
308 should be considered for future RT-qPCR experiments with *L. stagnalis*.
309

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313

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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.

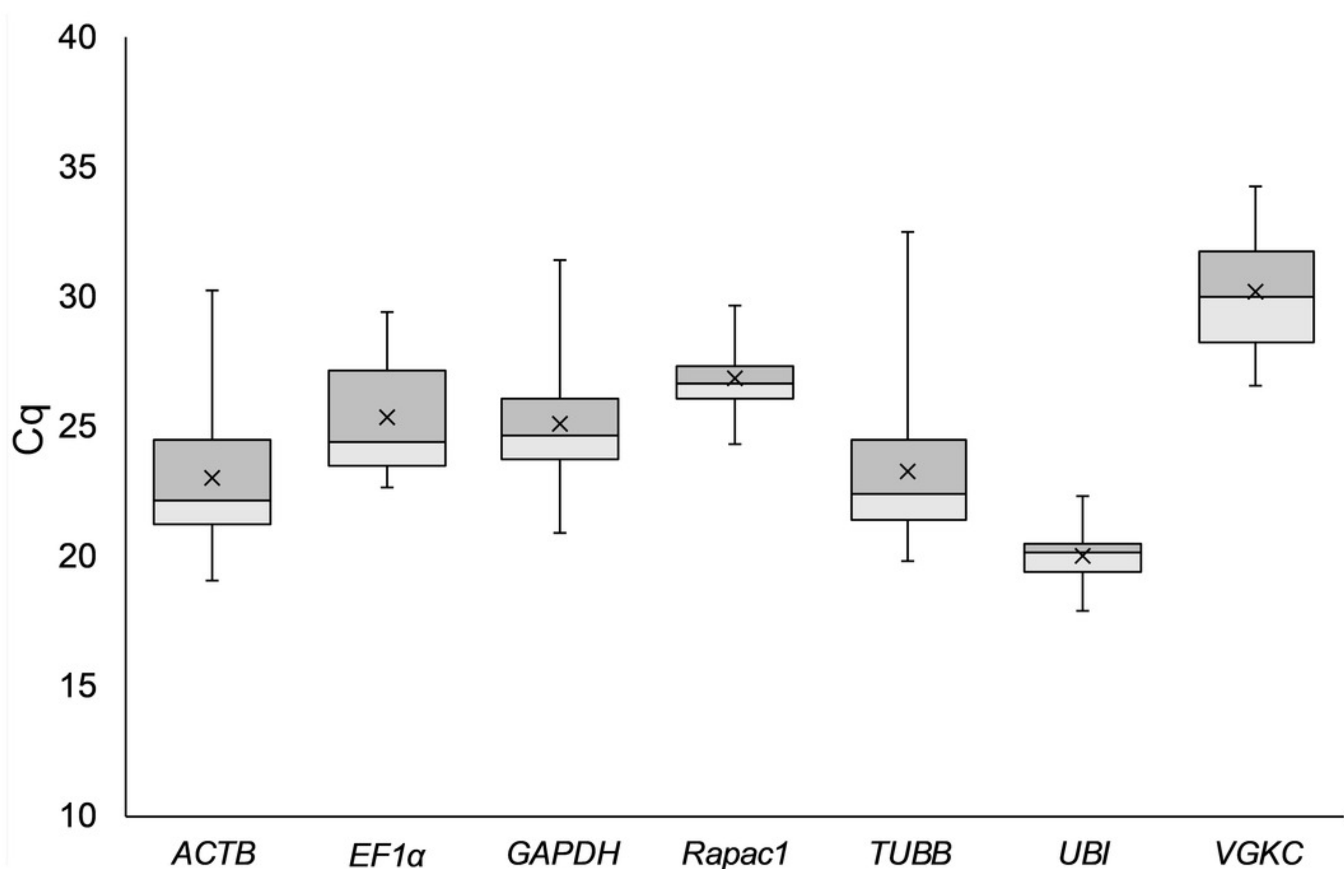
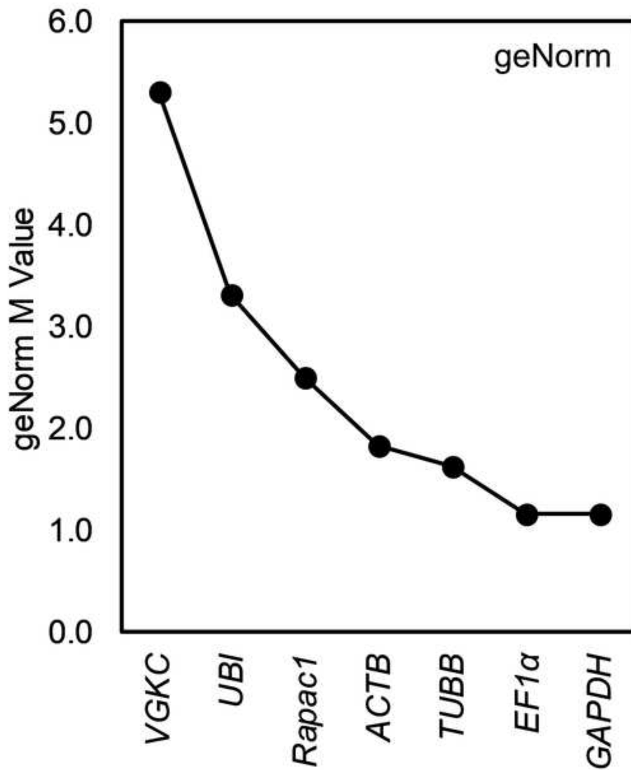
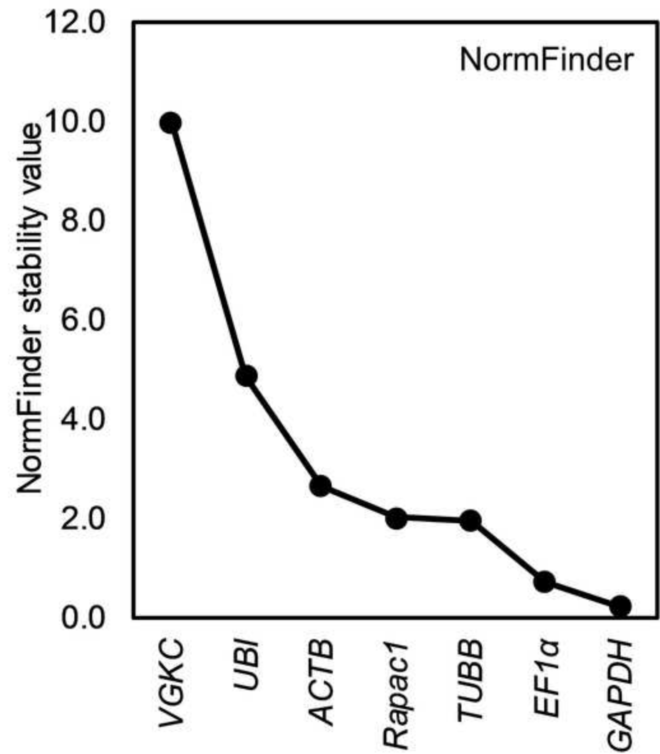


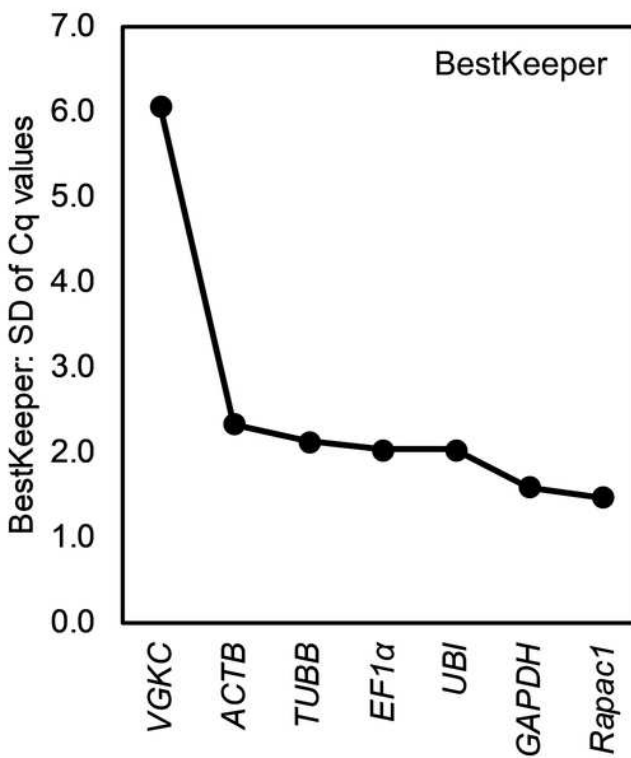
Figure 2

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.


geNorm
geNorm M Value
6.0
5.0
4.0
3.0
2.0
1.0
0.0
VGKC
UBI
Rapac1
ACTB
TUBB
EF1α
GAPDH


NormFinder
NormFinder stability value
12.0
10.0
8.0
6.0
4.0
2.0
0.0
VGKC
UBI
ACTB
Rapac1
TUBB
EF1α
GAPDH


BestKeeper
BestKeeper: SD of Cq values
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
VGKC
ACTB
TUBB
EF1α
UBI
GAPDH
Rapac1
Less stable More stable

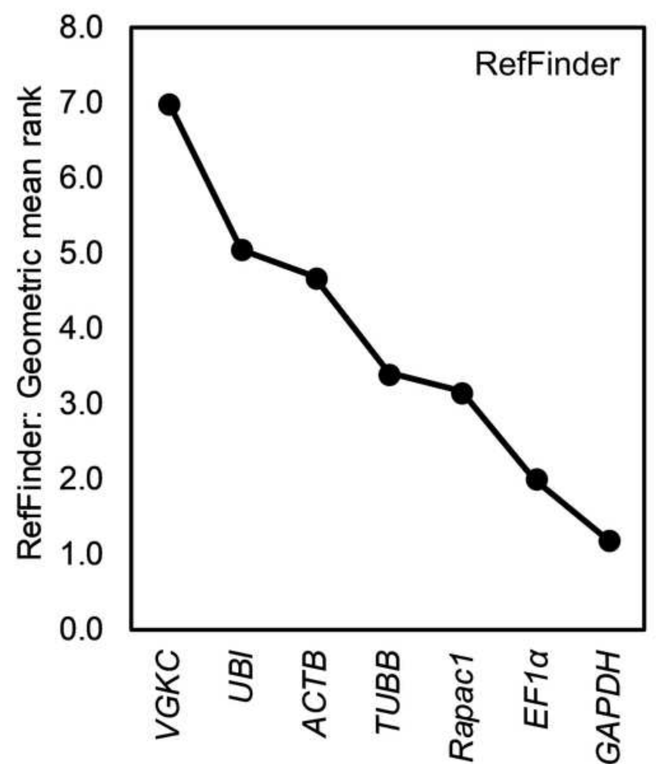

RefFinder
RefFinder: Geometric mean rank
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
VGKC
UBI
ACTB
TUBB
Rapac1
EF1α
GAPDH
Less stable More stable

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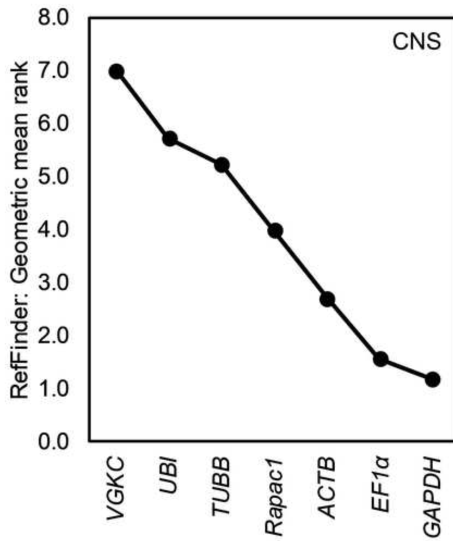
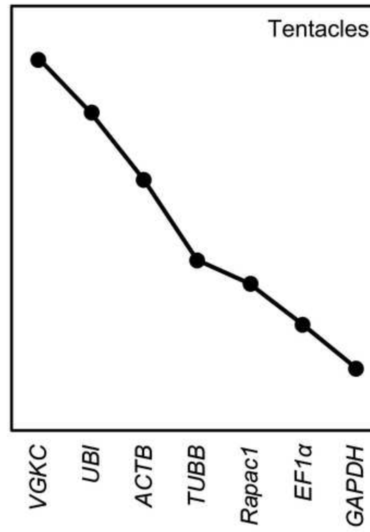
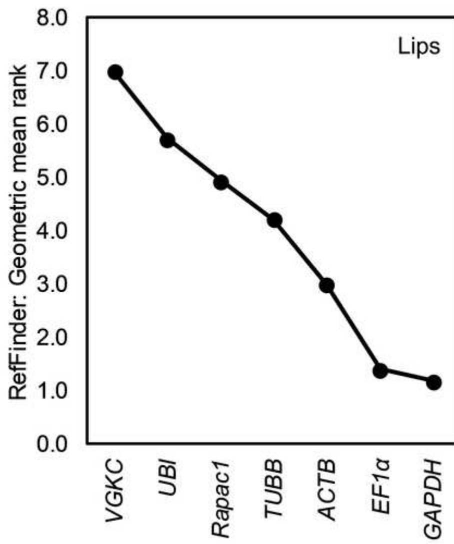
Figure 3

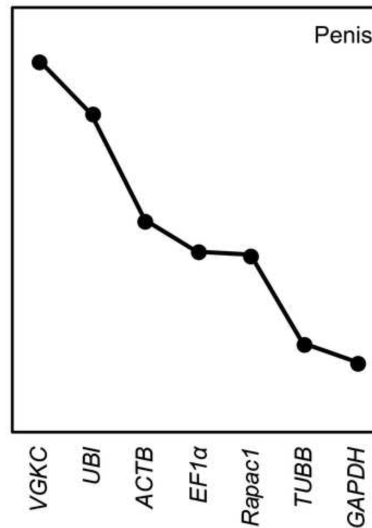
RefFinder comprehensive rankings for all candidate reference genes by tissue.

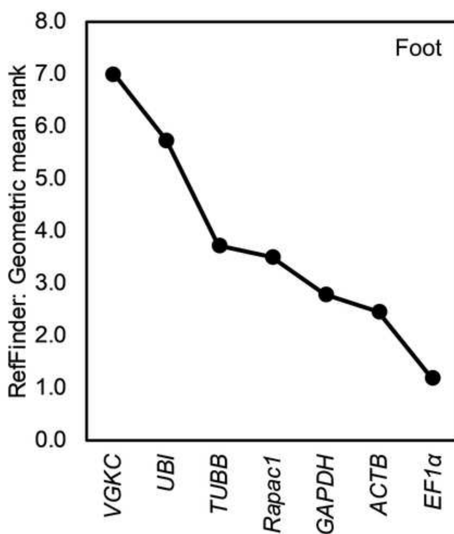
RefFinder calculates rankings as the geometric mean of the rankings assigned by geNorm, NormFinder and BestKeeper. Genes are ranked in order from the least stable to the most stable in each panel.


CNS
RefFinder: Geometric mean rank
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
VGKC
UBI
TUBB
Rapac1
ACTB
EF1α
GAPDH


Tentacles
VGKC
UBI
ACTB
TUBB
Rapac1
EF1α
GAPDH


Lips
RefFinder: Geometric mean rank
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
VGKC
UBI
Rapac1
TUBB
ACTB
EF1α
GAPDH


Penis
VGKC
UBI
ACTB
EF1α
Rapac1
TUBB
GAPDH


Foot
RefFinder: Geometric mean rank
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
VGKC
UBI
TUBB
Rapac1
GAPDH
ACTB
EF1α
Less stable More stable

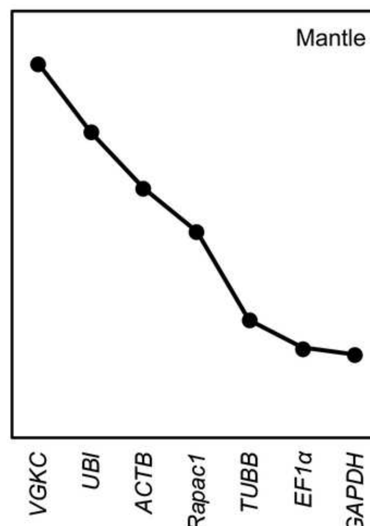

Mantle
VGKC
UBI
ACTB
Rapac1
TUBB
EF1α
GAPDH
Less stable More stable

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Figure 4

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.

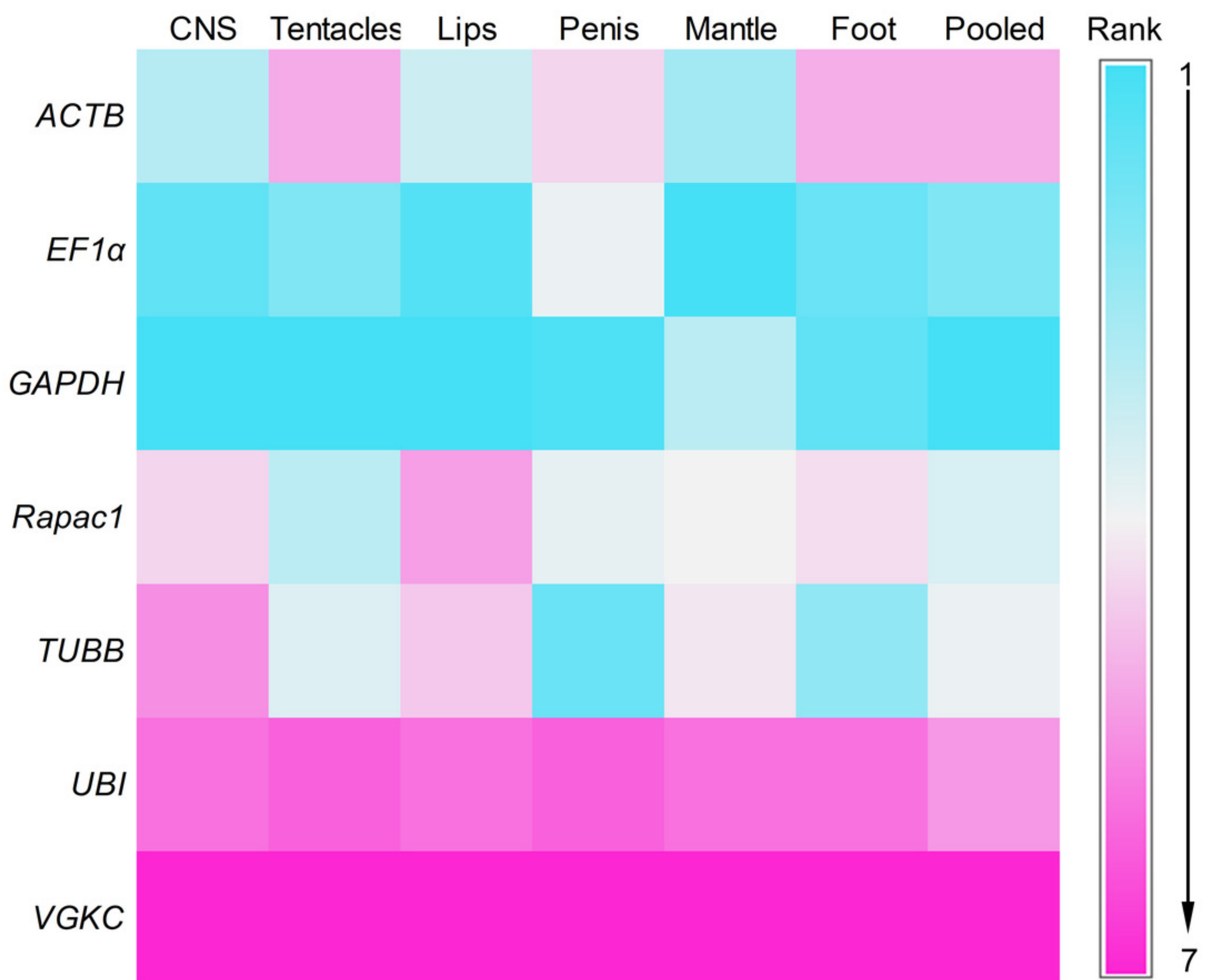

Table 1 (on next page)

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.

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1 **Table 1. Description of all primers used to amplify candidate reference genes in *Lymnaea stagnalis*.**

Gene	Primer Sequence (5' to 3')	Function	Product Size (bp)	Tm (˚C)	Efficiency	r^2	Accession
Lst-ACTB	For [AGGCCAACAGAGAAAAGA] Rev [AGATGCGTACAGAGAGAG]	Cell structure and motility	97	56	2.12	0.999	KX387883
Lst EF1α	For [ACCACAACTGGCCACTTGATC] Rev [CCATCTCTTGGGCCTCTTTCT] Probe [CGCTCATCAATACCACCACAC]	Delivery of tRNA for protein synthesis	85	59	2.00	0.998	MH687364
Lst-GAPDH	For [CAACAACCGACAAAGCAA] Rev [CATAACAAACATAGGGGCA]	Carbohydrate metabolism	93	55	1.82	0.988	MH687363
Lst-Rapac1	For [GGCTCTTTCTTTCCCTTTGT] Rev[TTCCTGCTCTTCTTGCGT]	Cellular trafficking	124	58	1.82	0.989	MH687365
Lst-TUBB	For [GGCTAGGGGATGAAGATGA] Rev [AGGATGAGGGTGAATTTGA]	Microtubule element – cell structure	130	56	1.80	0.993	KX387887
Lst-UBI	For [GTATTGTGGTGCTGGTGTTTT] Rev[GCTTCCTCCTCTGGTTTGT]	Regulate protein function	105	59	1.94	0.993	MH687367
Lst-VGKC	For [TGGCTTCCTGCTTCTCTGT] Rev[GCTTCTGTCGTTGTTTTGCT]	Maintenance of cell membrane potential	99	60	1.82	0.997	MH687366

2 Primers labeled "For" are forward primers and primers labeled "Rev" are reverse primers, all sequences are written in the 5' to 3'
3 direction. "Probe" represents the sequence of the hydrolysis probe used with the EF1α primer set. The amplification efficiency was
4 determined from each reference gene primer set following RT-qPCR with five 1:5 serial dilutions of total RNA from 100 ng.
5

Table 2(on next page)

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.

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

	Lst-ACTB		*Lst-EF1α*		*Lst-GAPDH*		*Lst-Rapac1*		*Lst-TUBB*		*Lst-UBI*		*Lst-VGKC*	
Tissue	**Cq Mean**	**SEM**	**Cq Mean**	**SEM**	**Cq Mean**	**SEM**	**Cq Mean**	**SEM**	**Cq Mean**	**SEM**	**Cq Mean**	**SEM**	**Cq Mean**	**SEM**
CNS	22.04	0.23	23.85	0.21	24.07	0.16	26.27	0.13	21.48	0.22	19.75	0.12	29.32	0.43
Tentacle	21.71	0.42	24.93	0.50	25.17	0.38	27.40	0.22	22.47	0.50	20.47	0.23	30.10	0.49
Lips	21.47	0.35	23.89	0.19	24.06	0.17	25.96	0.21	21.62	0.20	19.23	0.22	30.19	0.49
Penis	22.66	0.32	25.42	0.46	24.72	0.36	27.42	0.26	23.01	0.29	20.52	0.16	31.43	0.56
Foot	24.78	0.85	27.43	0.67	26.62	0.82	27.00	0.29	26.31	0.89	20.41	0.22	29.72	0.40
Mantle	25.40	0.89	27.20	0.55	25.75	0.39	27.12	0.39	24.56	0.70	19.82	0.22	30.42	0.60

2 Means were calculated from six biological replicates, all with three technical replicates each.

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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, cytochromep450 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.

1 **Table 1. Summary of the stability rankings of reference genes from studies conducted in molluscs.**

Organism	Most stable gene(s)	Least stable gene(s)	Other Genes Tested	Reference
Gastropods				
Haliotis discus	*EF1α/RPL5*	*18S rRNA*	*ACTB, BGLU, CY, GAPDH, H2A, HPRT, SDHA, TUBB, UBC, CYP4*	Wan et al. 2011
Bellamya aeruginosa	*RPL7*	*ACTB*	*18S rRNA, EF1α, GAPDH, TUBB, H2A, DRP2*	Liu et al. 2016
Cepaea nemoralis	*EF1α/ACTB*	*GAPDH*	*DNARP, FIB3, GTP8, Rapac1, RNAP, TUBA, UBI, VGKC*	Affenzeller et al. 2018
Lymnaea stagnalis	*EF1α/GAPDH*	*VGKC*	*ACTB, TUBB, UBI, Rapac1*	Present study
Cephalopods				
Octopus vulgaris	*TUBA/UBI*	*18S rRNA*	*16S rRNA, ACTB, EF1α, TUBA*	Sirakov et al. 2009

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Octopus vulgaris	*UBI*	*ACTB*	*18S rRNA, EF1α, GAPDH, TUBA*	García-Fernández et al. 2016
Bivalves				
Ostrea edulis	*EF1α/GAPDH*	*ACTB*	*UBI, RPL5*	Morga et al. 2010
Mytilus edulis	*EF1α/18S rRNA*	*ACTB*	*28S rRNA, TUBA, HEL*	Cubero-Leon et al. 2012
Mytilus galloprovincialis	*EF1α*	*ACTB*	*18S rRNA, TUBA*	Moreira et al. 2015
Ruditapes philippinarum	*TUBA*	*ACTB*	*18S rRNA, EF1α*	Moreira et al. 2015
Crassostrea gigas	*EF1α*	*RPS18*	*ACTB, ARF1, GAPDH, HNRPQ, UBC*	Huan et al. 2016

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Mytilus galloprovincialis	*GAPDH/RPS4*	*NAD4/18S rRNA*	*ACTB, COX1, GAPDH, RPS27, TIF5A*	Martínez-Escauriaza et al. 2018

2 ARF1, adp-ribosylation factor 1; BGLU, beta-glucuronidase; COX1, cytochrome c oxidase subunit 1; CY, cyclophilin; CYP4,
3 cytochromep450 family 4; DNARP, DNA repair protein; DRP2, DNA-directed RNA polymerase II; FIB3, fibronectin type III domain
4 containing protein; GTP8, GTP-binding protein; H2A, histone H2A; HEL, RNA helicase; HNRPQ, heterogeneous nuclear
5 ribonucleoprotein q; HPRT, hypoxanthine phosphoribosyltransferase 1; NAD4, NADH dehydrogenase subunit 4; RNAP, RNA-
6 directed DNA polymerase; RPL5, ribosomal protein L5; RPL5, ribosomal protein L7; RPS4, 40S ribosomal protein S4; RPS27, 40S
7 ribosomal protein S27; SDHA, succinate dehydrogenase; UBC, ubiquitin-conjugating enzyme.
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