

1 **A new set of endogenous control genes for use in quantitative real-time PCR**
2 **experiments show that formin *Ldia2^{dex}* transcripts are enriched in the early**
3 **pond snail embryo**

4 Harriet F. Johnson* and Angus Davison

5 *School of Life Sciences, University Park, University of Nottingham, NG7 2RD. UK*

6 *Correspondence: Harriet Johnson; e-mail: harrietjohnson@gmail.com*

7 Running head: control genes for qRT-PCR in pond snails

1 **ABSTRACT**

2 Although the pond snail *Lymnaea stagnalis* is an emerging model organism for
3 molecular studies in a wide variety of fields, there are a limited number of verified
4 endogenous control genes for use in quantitative real-time PCR (qRT-PCR). As part
5 of larger study on snail chirality, or left-right asymmetry, we wished to assay gene
6 expression in pond snail embryos. We therefore evaluated six candidate control
7 genes, by comparing their expression in three tissues (ovotestis, foot, and embryo)
8 and across three programs (geNorm, Normfinder and Bestkeeper). The specific
9 utility of these control genes was then tested by investigating the relative expression
10 of six experimental transcripts, including the formin *Ldia2*, a gene that has been
11 associated with chiral variation in *L. stagnalis*. All six control genes were found to be
12 suitable for use in the three tissues tested. Of the six experimental genes, it was
13 found that all were relatively depleted in the early embryo compared with other
14 tissues, except the formin gene *Ldia2*. Instead, transcripts of the wild type *Ldia2^{dex}*
15 were enriched in the embryo, whereas a non-functional frameshifted version *Ldia2^{sin}*
16 was severely depleted. These differences in *Ldia2^{sin}* expression were less evident in
17 the ovotestis and not evident in the foot tissue, possibly because nonsense-mediated
18 decay is obscured in actively transcribing tissues. This work therefore provides a set
19 of control genes that may be useful to the wider community, and illustrates how they
20 may be used to assay differences in expression in a variety of tissues.

1 INTRODUCTION

2 The pond snail *Lymnaea stagnalis* is a hermaphrodite, pulmonate snail which is
3 increasingly used in a wide range of research areas including ecology, evolution,
4 development, neuroscience, behaviour, parasitology and sexual selection. Due to
5 the species perhaps predominant prior use as a model system in neuroscientific
6 studies, many of the earlier published molecular studies were confined to the central
7 nervous system (e.g. Feng *et al.*, 2009). More recently, molecular studies have come
8 from different fields, including especially ecotoxicology and biomineralisation
9 (Bouetard *et al.*, 2012; Hohagen & Jackson, 2013). An unannotated draft genome
10 sequence is available (Davison *et al.*, 2016), and there is a collaborative effort
11 underway to produce a publically available, high-quality, annotated genome
12 sequence (Genoscope-CEA, de la Recherche à l'Industrie, France).

13 In the past few years, *L. stagnalis* snails have also become an important
14 organism in the study of left-right asymmetry, because the species exhibits
15 genetically tractable variation in chirality (Kuroda *et al.*, 2009; Shibazaki, Shimizu &
16 Kuroda, 2004). This recently culminated in our finding that a disabling frameshift
17 mutation in one copy of a diaphanous-related formin *Ldia2* is associated with early
18 symmetry breaking in the developing embryo (Davison, *et al.*, 2016).

19 In preparing that work, we decided that it was necessary to design a new set
20 of control genes to use with quantitative real-time PCR (qRT-PCR) in *L. stagnalis*.
21 Specifically, as we needed to measure the expression of cytoskeletal genes in *L.*
22 *stagnalis*, then this precluded the use of genes such as actin and tubulin as
23 appropriate endogenous controls, because they are themselves cytoskeletal genes.
24 Unfortunately, many of the previously published qRT-PCR studies on *L. stagnalis*

1 either used ribosomal RNA (rRNA), actin or tubulin genes as endogenous controls
2 (Bavan *et al.*, 2012; Bouetard *et al.*, 2013; Carter *et al.*, 2015; Hatakeyama *et al.*,
3 2013; Lu & Feng, 2011; Ribeiro *et al.*, 2010; van Kesteren *et al.*, 2006; van Nierop *et*
4 *al.*, 2006). rRNA genes are potentially problematic because the over-abundance of
5 rRNAs relative to the target mRNA sequence can lead to problems in accurate
6 normalisation, and in any case, rRNA is transcribed through an independent pathway
7 from mRNA and therefore not regulated in the same manner (Radonic *et al.*, 2004).
8 More generally, it is now widely accepted that there are no universal endogenous
9 control genes, and each gene intended for use as an endogenous control should
10 ideally be validated as consistently expressed across all experimental conditions.

11 Therefore, we aimed to develop and test a new set of endogenous genes as
12 controls, for use in our study, but also for subsequent use by the wider community,
13 just as has been the case in some other species (Hibbeler, Scharsack & Becker,
14 2008; Li *et al.*, 2017; Olias *et al.*, 2014; Sirakov *et al.*, 2009). These new control
15 genes were then used to compare expression between different chiral genotypes of
16 snail, and between different tissues.

1 **METHODS**

2 *Sample preparation*

3 Three separate tissues were used: single-cell embryo, ovotestis (hermaphrodite
4 gonad) and foot, all from laboratory reared individuals of *L. stagnalis*. Total RNA was
5 extracted from pooled embryos from a single individual using the RNeasy micro kit
6 (Qiagen), including DNase (Qiagen) treatment, yielding approximately 0.5 ng total
7 RNA per embryo (Johnson, 2016). Ovotestis and foot tissue samples were removed
8 from individual adult snails and snap frozen using a dry ice/ethanol slurry. Total RNA
9 was immediately extracted from them using TRI Reagent® solution (Applied
10 Biosystems).

11 RNA quality and concentration were immediately assessed using both
12 agarose gel electrophoresis and a NanoDrop spectrophotometer, with samples then
13 stored at -80°C. To establish a set of endogenous gene controls, it would be
14 advisable to use precisely the same quantities of mRNA in each complementary
15 DNA (cDNA) synthesis reaction. We therefore aimed to use 500 ng total RNA for
16 each sample, using Superscript III reverse transcriptase (Invitrogen) and random
17 primer mix (NEB). There was limited variation (Supplementary Table 1 for full details)
18 for the foot tissue (n = 10, mean = 500 ng, S.D. = 2, range = 497-503 ng – beyond
19 the precision of measurement) and for the ovotestis (n = 9, mean = 499 ng, S.D. = 1,
20 range 497-500). Less RNA was available from the embryo, because 500 ng of
21 embryo RNA would have required ~1000 eggs from a single snail. Therefore, instead
22 of reducing the RNA to that of the lowest yielding (and possibly poorest quality
23 sample), we allowed 2.5 fold variation (n = 12, mean = 121 ng, S.D. 30, range 75-
24 189 ng) for this tissue.

1 Including individuals that were also used to compare expression of
2 experimental genes, there was slightly wider variation in initial starting RNA quantity
3 for both ovotestis (n = 36, mean = 505 ng, S.D. 31, range 375-572; only one sample
4 less than 463 ng) and embryo (n = 17, mean = 135 ng, S.D. 40, range 75-233 ng)
5 (Supplementary Table 1).

6 Serial dilutions were performed independently for each standard curve
7 experiment. Aliquots were then made of the experimental working concentration
8 dilutions of cDNA to reduce freeze-thaw cycles. All cDNA samples were gently
9 vortexed before use and prior to each serial dilution step.

10 *Primer design*

11 Using transcriptomic resources of 1-2 cell stage *L. stagnalis* embryos (Liu *et al.*,
12 2014), six genes were selected as potential endogenous controls, all with well-
13 characterised gene function. These were short-chain specific acyl-CoA
14 dehydrogenase (*Lacads*), elongation factor 1-alpha (*Lef1a*), histone protein, H2A
15 (*Lhis2a*), 60S ribosomal protein L14 (*Lrpl14*), ubiquitin-conjugating enzyme E2
16 (*Lube2*), and 14-3-3 protein zeta (*Lywhaz*). Primer pairs were then designed using
17 Primer 3 (Rozen & Skaletsky, 2006), aiming for a T_m range within 2°C, and
18 amplicon product sizes between 110-130bp, including GC clamps where possible.
19 All primer pairs were intron-spanning, with the primers on exon/intron boundaries,
20 where possible, to minimise problems with accidental genomic DNA carry over. To
21 initially verify the primers, produced by IDT, a standard PCR was used alongside a
22 genomic DNA control sample and the products visualised on an agarose gel.
23 Additionally, the specificity of the amplicons of all six primer pairs was verified
24 through Sanger sequencing.

1 *Primer specificity and amplification efficiency*

2 Primer efficiencies for each primer pair were calculated via standard curve qRT-PCR
3 experiments using the Applied Biosystems 7500 fast system v2.3 and the same
4 cycling parameters (below). Five standardised concentrations were used with an
5 additional negative control (PCR grade water). Five-step serial cDNA dilutions were
6 performed using molecular grade water and a dilution factor of 1:5. Primer
7 efficiencies for all six endogenous control gene primer pairs were estimated using
8 the same reference sample, created from pooling cDNA samples. Average primer
9 efficiencies for each primer pair were then calculated via the arithmetic mean of a
10 minimum of two successful standard curve experiments. A standard curve
11 experiment was considered successful if it produced a R^2 value of >0.98 . Values
12 from the lowest concentration dilutions were omitted if they dramatically reduced the
13 amplification efficiency or R^2 value of an experiment. The range of dilutions included
14 in the standard curve experiment indicates the limits of acceptable working
15 concentration/dilution factor for an experimental comparative qRT-PCR assessment.

16 Cycle threshold (Cq) values were obtained from qRT-PCR experiments using
17 the ABI 7500 fast system v2.3. Each reaction contained 5 μ l of Primer Design's fast
18 SYBR® green master mix, 0.5 μ l forward and reverse primer (4 μ M), 1.5 μ l PCR
19 grade water and 3 μ l of cDNA. All samples were used at a 1:30 dilution of the original
20 cDNA concentration. Mastermixes were prepared for each target gene experiment
21 and a temperature melt curve step was included at the end of all qRT-PCR
22 reactions. Thermocycling parameters were as follows: 95°C for 20, 95°C for 3
23 seconds, 60°C for 30s (data collection, Cq), then 39 cycles between steps 2 and 3;
24 this was followed by 95°C for 15 seconds, 60°C for 60 seconds, a slow temperature
25 ramp 1% (data collection; temperature melt curve), 95°C for 15 seconds, 60°C 15

1 seconds; temperature melt curves indicated that a single specific product was
2 produced in all cases.

3 *Normalising control software*

4 Three methods were used to assess the same qRT-PCR data, all of which run as
5 macros within Microsoft Excel 2003. BestKeeper used raw Cq values (Pfaffl *et al.*,
6 2004), whereas NormFinder (Andersen, Jensen & Orntoft, 2004) and geNorm
7 (Vandesompele *et al.*, 2002) required linearised Cq values. Efficiency-corrected
8 linearised relative Cq values were calculated for each sample using the Pfaffl method
9 (Hellemans *et al.*, 2007). BestKeeper ran entirely from raw Cq values and corrected
10 for amplification efficiency via the inbuilt formulas within the macro, via the manually-
11 input amplification efficiency values.

12 *Snail lines and tissues*

13 Variation in the left-right asymmetry of snails, or chirality, is under the control of a
14 single maternally expressed locus. In *L. stagnalis*, maternal *D* alleles dominantly
15 determine a clockwise (“dextral”) twist in offspring. For our experiments we created a
16 single near-isogenic line of snails (>99% inbred) that was still variable for the chirality
17 locus, by repeated backcrossing (Davison, *et al.*, 2016). From this line, separate
18 homozygous dextral (*DD*) and sinistral (*dd*) lines were produced. Heterozygote (*Dd*)
19 snails were then derived by crossing individuals from the near-isogenic lines. qRT-
20 PCR data was generated for *DD*, *Dd* and *dd* genotypes using both embryo and
21 ovotestis tissue; however as resources were limited, only *DD* and *dd* genotypes were
22 sampled for foot tissue.

23 *Relative expression of cytoskeletal genes*

1 Previously, we reported finding that tandemly duplicated, diaphanous-related formin
2 genes, *Ldia1* and *Ldia2*, are perfectly associated with variation in chirality of the
3 pond snail, and that the sinistral-derived version of *Ldia2* contains a disabling
4 frameshift mutation, which results in much reduced levels of *Ldia2* mRNA in the
5 embryo (Davison, *et al.*, 2016). To further explore changes in expression between
6 genotypes (*DD*, *Dd*, *dd*) and tissues (single-cell embryo, foot, ovotestis), the relative
7 expression of *Ldia1* and *Ldia2* was tested against the validated endogenous control
8 genes.

9 As above, primer pairs were designed using Primer 3 with the same
10 conditions (Table 1). However, because of the high sequence identity between *Ldia1*
11 and *Ldia2*, it was not possible to design intron-spanning PCRs for these loci. Instead,
12 primer pairs were designed in the 3'UTR, because this region was most variable
13 between copies, *Ldia1 3'UTR* and *Ldia2 3'UTR*, in addition to a primer pair in the
14 open reading frame, *Ldia2 ORF*. Four other genes were also tested, including the
15 cytoskeletal genes furry *Lfry* and fat-like cadherin *Lfat*, both tightly linked to *Ldia1/2*
16 on the same chromosome, as well as unlinked actin-related proteins subunits 1a and
17 3, *Larp2/3-1a* and *Larp2/3-3*.

18 Relative expression of these six genes (seven primer pairs) was tested
19 against the endogenous controls by calculating the Normalised Relative Quantity
20 (NRQ) values from the average Cq value of each sample using the Pfaffl method
21 (Hellemans, *et al.*, 2007; Pfaffl, 2001; Pfaffl, *et al.*, 2004), relative to a single
22 standard *DD* snail. Experimental samples were performed in triplicate repeat and
23 negative controls in duplicate repeat for each of the six genes.

1 For each sample, first the relative quantity per target gene (ΔCq target) was
2 calculated by subtracting the average Cq value of the sample from that of the
3 calibrator sample. This ΔCq value was then corrected for amplification efficiency (E)
4 by multiplying ΔCq to the base percentage amplification efficiency (represented as a
5 value between 1 and 2). The efficiency-corrected relative quantities were then
6 normalised to the endogenous control genes by dividing by the geometric mean
7 (geoM) of the efficiency corrected delta Cq values calculated for each of the control
8 genes (ΔCq ref) in the same manner as described above.

9 To measure the relative expression of cytoskeletal genes between genotypes
10 *within* the same tissue, NRQ values were normalised to the geometric mean of the
11 three endogenous control genes, using *Lhis2a*, *Lube2* and *Lywhaz* for embryo and
12 foot, and *Lhis2a*, *Lube2* and *Lrpl14* for ovotestis. The standard was cDNA made
13 from the same tissue, using a single *DD* snail. To make comparisons *across* different
14 tissues, NRQ values were normalised to the geometric mean of the two endogenous
15 control genes quantified in all tissues, *Lhis2a* and *Lube2*. The standard was the
16 exactly the same sample for each analysis, cDNA made from pooled ovotestis RNA
17 of mixed genotype.

1 RESULTS

2 *Primer specificity and amplification efficiency*

3 All control primer pairs demonstrated amplification efficiencies between 1.906 and
4 2.115 with R^2 values exceeding 0.98 (Table 1). All primers demonstrated acceptable
5 amplification efficiencies in dilutions up to 1:150 (0.67%) of the full concentration.

6 The working concentration of a 1:30 dilution that was used in the subsequent qRT-
7 PCR experiments fell well within these limits.

8 *Comparing normalising control software*

9 Summaries of the top genes to use for each tissue are shown in Table 2. In fact, all
10 genes and all combinations were acceptable for use. *Lef1a* was consistently found to
11 be the least stable gene in all tissues but was still recommended in some analyses
12 (Table 2).

13 In full detail (Table 3), in the embryo geNorm placed *Lhis2a* and *Lube2* as the
14 most stable pair of genes, with a combined stability score of 0.196. The inclusion of
15 any number of the genes provided a V score of <0.15 , indicating that the
16 combination of genes will provide a reliable normalisation factor (PrimerDesign
17 2014). The lowest (best) V score was achieved with the inclusion of the five genes
18 *Lhis2a*, *Lube2*, *Lrpl14*, *Lacads* and *Lywhaz*. In the foot, geNorm placed *Lywhaz* and
19 *Lube2* as the most stable pair of genes with a combined stability score of 0.217. The
20 inclusion of any number of the genes provided a V score of <0.15 , although the
21 lowest V score was achieved with the inclusion of the four genes *Lywhaz*, *Lube2*,
22 *Lhis2a* and *Lacads*. In the ovotestis, geNorm placed *Lrpl14* and *Lube2* as the most
23 stable pair of control genes with a combined score of 0.250. *Lhis2a* bore the lowest
24 M score of all the target genes, at 0.360, yet it was placed fourth in the combined

1 stability score. Again, the inclusion of any number of the genes provided a V score of
2 <0.15, although the lowest V score was achieved with the inclusion of the three
3 genes; *Lrpl14*, *Lube2* and *Lywhaz*.

4 NormFinder outputs the most stable pair and most stable individual gene.
5 *Lhis2a* was the most stable gene in the embryo (stability value of 0.058; Table 3), but
6 the best combined pair was *Lacads* and *Lube2* (0.047). In the foot, *Lywhaz* was
7 identified as the most stable gene (0.074) and was paired with *Lube2* (combined
8 stability 0.066). In the ovotestis, *Lhis2a* was most stable (0.124), but the best
9 combined pair of genes was *Lef1a* and *Lywhaz* (0.083), despite the fact that *Lef1a*
10 presented the poorest (highest) individual gene stability value (0.243). As with
11 geNorm, the embryo analyses yielded the least variable scores. *Lef1a* was found to
12 be the least stable or second least stable individual gene in all tissues. In all
13 analyses, the stability value of the best combined pair of genes was lower than that
14 of any individual gene stability score.

15 The BestKeeper program provides two measures of gene stability, with a low
16 SD (<1) and a high r value indicating a more stable control gene; additionally a
17 statistically significant correlation with the BK index (generated from all data), is used
18 to show that the data is in keeping with that expected across samples/low
19 variability. In the embryo, the gene ranked as most stable according to SD was
20 *Lhis2a* (0.408; Table 3), whereas the least stable gene was *Lef1a* (0.577). Every
21 gene in the embryo analysis resulted in a highly significant positive correlation with
22 the BestKeeper index ($P = 0.001$). *Lhis2a* demonstrated the highest correlation, with
23 an r value of 0.979, and *Lywhaz* the lowest with an r value of 0.900. In the foot,
24 *Lrpl14* was ranked as most stable according to SD (0.500), whereas the least stable

1 gene was *Lhis2a* (0.947). With the exception of *Lef1a* in the ovotestis, every
2 gene/tissue combination showed a significant correlation with the BK index.

3 *Relative expression of cytoskeletal genes*

4 Relative expression of *Ldia2* transcripts depends upon the genetic background of the
5 mother. Thus, levels of *Ldia2* transcripts in embryos derived from a genetically
6 sinistral mother *dd* were 0.006 (*Ldia2* 3'UTR, 0.6%) or 0.03 (*Ldia2* ORF, 3%) relative
7 to embryos from a wild-type *DD* mother (Figure 1; Tables 4 and 5); levels of the
8 same transcripts in offspring of a heterozygote mother *Dd* were about half that of
9 wild-type, 0.56 (*Ldia2* 3'UTR, 0.56%) or 0.48 (*Ldia2* ORF, 0.48%). Notably, the
10 relative differences in expression of *Ldia2* transcripts were much less striking in
11 ovotestis, though still significantly lower, 0.81 (*Dd*) and 0.69 (*dd*) using *Ldia2* 3'UTR
12 and 0.80 (*Dd*) and 0.62 (*dd*) using *Ldia2* ORF; in foot tissue, there were no
13 significant differences in expression between *Ldia2* transcripts from snails of different
14 genotype (Figure 1; Tables 4 and 5). In comparison, there were few significant
15 differences in the expression of the other genes with the exception of *Larp2/3-3*
16 (*DD:Dd*, and *Dd:dd*) and *Larp2/3-1a* (*Dd:dd*) in the embryo.

17 Using homozygous dextral (*DD*) snails, all of the tested gene transcripts were
18 relatively depleted in the single-cell embryo compared against ovotestis and foot,
19 except *Ldia2*, which was enriched (Figure 2; Table 6). *Larp*, *Lfat* and *Lfry* transcripts
20 were reduced to ~0.03 to 0.27 of the level in embryo compared to ovotestis, and
21 ~0.11 to 0.38 when comparing foot to single-cell embryo. In comparison, levels of
22 *Ldia2* expression were ~1.27 to 2 times higher in the single-cell embryo compared to
23 the ovotestis and ~2.8 times higher when compared to the foot tissue.

1 DISCUSSION

2 Individually, all six gene targets were found to provide stable endogenous controls
3 across all tissues. However, the best individual gene and combination of genes
4 differed between tissues used and analysis program (Table 2). As it is recommended
5 to use more than one control gene in combination in an experiment, then a tissue
6 specific analysis is advisable prior to the experiment proper. Whether adding a third
7 gene is worth the additional time and resources will depend on the individual
8 experiment and the extent of the increase in stability gained.

9 *Genes to use as endogenous controls in different tissues*

10 Within the embryo, all three algorithms ranked *Lhis2a* as the most stable single
11 gene, but there was less consensus for the rankings of the remaining endogenous
12 controls. Generally, *Lhis2a*, *Lrpl14* and *Lube2* were in the top three most stable
13 genes across software and tissue (Tables 2 and 3). For the foot tissue analyses, due
14 to the agreement of GeNorm and Normfinder, *Lywhaz* and *Lube2* might be
15 recommended as endogenous normalising controls. In comparison, for the ovotestis
16 both geNorm and BestKeeper showed that the use of *Lrpl14* and *Lube2* might be
17 recommended, with the inclusion of a third gene, *Lywhaz*, indicating the most stable
18 combination of genes.

19 *Lef1a* was consistently ranked least stable, interesting because it has been a
20 common choice by others as an endogenous control (Foster, Lukowiak & Henry,
21 2015; van Nierop, *et al.*, 2006). However, we found that it is still acceptable for use,
22 just not necessarily the gene of choice. The reason for the relatively poor
23 performance may be due to a low level of expression rather than variable
24 expression, indicated in the amplification efficiency experiments (Table 1). *Lef1a*

1 may thus provide a reliable endogenous control gene when using an increased
2 cDNA concentration.

3 Compared to the other tissues assessed, the embryo was found to be least
4 variable (Figure 2). There are many reasons why some tissues may be more
5 variable than others. In our experiments, it was difficult to temporally control the
6 extraction of the ovotestis (e.g. time since egg-laying), and especially to make sure
7 that it was free of contaminating hepatopancreas. In comparison, the embryos were
8 from a clean and temporally controlled sample.

9 All three analytical programs used here provided a unique aspect of the data
10 analysis. geNorm provided a measure of the optimum number of genes to include in
11 the analysis and an advised cut-off value (V , <0.15) for an acceptable endogenous
12 control gene combination. BestKeeper output a quotable measure of SD for each
13 gene and a statistical measure of the relatedness of gene expression. Finally,
14 NormFinder provided valuable information on the experimental design; calculating
15 variation created both within and between experimental groups and importantly
16 provides an alternative to pairwise comparison methods.

17 *Comparing expression in different tissues*

18 Previously, we used qRT-PCR to show the formin *Ldia2* shows significant fold-
19 change differences in the quantity of mRNA transcripts between different chirality-
20 associated genotypes (Davison, *et al.*, 2016). Here we showed that the cytoskeletal
21 genes, including *Ldia1*, are substantially depleted in the embryo, except for dextral-
22 derived alleles of the formin, *Ldia2^{dex}*, which are substantially enriched (Figure 2). In
23 comparison, the frameshifted version, *Ldia2^{sin}*, was severely depleted in single-cell
24 embryos (Figure 1), but these differences were less evident in the ovotestis and not

1 evident in the foot tissue (Figure 2). This may partly due to lower levels of
2 expression, especially in the foot tissue, but mainly because the ability to detect the
3 dynamics of nonsense mediated decay of RNA may be obscured in actively
4 transcribing tissues.

5 As the cellular processes associated with variations in *L. stagnalis* chirality are
6 predominantly cytoskeletal (Davison, *et al.*, 2016; Shibazaki, *et al.*, 2004; Tee *et al.*,
7 2015), this work further emphasises the potential pitfalls of using the commonly
8 employed endogenous control genes, actin or tubulin, without adequate testing of
9 their expression stability. It also suggests that *Ldia1/Ldia2* have different roles during
10 development, despite the close sequence similarity, and that *Ldia2* may be
11 particularly critical in early development, given the relatively enriched levels of
12 transcript present in the single cell embryo.

13 Comprehensive studies of nonsense-mediated decay have not been
14 performed in molluscs. However, in nonsense-mediated decay studies, from yeast to
15 mammals, decay has been observed in both a 5' to 3' and 3' to 5' direction of the
16 mRNA, originating from either the 3' end or exon-exon boundaries (Karousis, Nasif &
17 Mühlemann, 2016; Lykke-Andersen & Jensen, 2015). The variation in starting
18 position of nonsense-mediated decay limits interpretation of the differences between
19 the reduction *Ldia2* in the 3' UTR and ORF. However, the frameshift in *Ldia2^{sin}*
20 transcripts should be present in all tissues, and therefore the resulting nonsense-
21 mediated decay would be expected to be evident in all tissues. The lack of significant
22 quantitative differences in the foot tissue suggests that nonsense-mediated decay
23 may be obscured in actively transcribing tissues. As transcription does not begin in
24 *L. stagnalis* before the 8-cell stage (Liu, *et al.*, 2014), so a single-cell embryo only
25 contains maternal mRNAs that are transcribed prior to oviposition; ovotestis is

1 presumably enriched for this same material. Further experiments with later stage
2 embryos would presumably confirm this hypothesis.

3 *Limitations*

4 In seeking to establish a set of verified endogenous control genes for use in
5 quantitative real-time PCR (qRT-PCR), it would be advisable to use a consistent
6 starting quantity of mRNA in each cDNA synthesis reaction. In this study, our aim
7 was to use 400-500 ng total RNA in each starting reaction, but reality meant that
8 there was some variation, minor for the foot and ovotestis tissue but more significant
9 for the embryo, also using less RNA for the latter due to the limited yield of RNA from
10 a single cell embryo.

11 While the results show that the primer-pairs are efficient over several orders
12 of magnitude, the relative ratios of the endogenous controls and tested genes should
13 not differ, irrespective of the starting quantity of RNA that was used as input. Any
14 minor variation should not therefore impact upon measured relative expression of
15 experimental genes. However, in terms of establishing the endogenous controls in
16 the first place, it would have been preferable to use samples with less variation,
17 especially for the embryo, and to use a more accurate method to measure RNA over
18 the NanoDrop, such as a Qubit fluorimeter.

19 The stability analyses using foot and ovotestis may be more reliable than
20 those using embryo, because of the greater variation in input RNA for embryo.
21 Nonetheless, it is worth noting that the tissue with the lowest starting quantity of RNA
22 and the most variation, embryo, showed the least variation in relative transcript
23 levels.

24 *Conclusions*

1 It was established that any of the six genes would provide acceptable endogenous
2 controls to standardise gene expression between chiral genotypes within any of the
3 three different tissues. These primers should therefore permit rapid verification of
4 endogenous controls suitable for use in qRT-PCR experiments assessing ovotestis,
5 foot and embryo tissue within and between chiral variants of *L. stagnalis*, which was
6 lacking previously.

7 **SUPPORTING INFORMATION**

8 **Supplementary Information 1.** Fasta format alignments of genomic sequences
9 against transcriptome and primer sequences for endogenous control genes and
10 cytoskeletal genes.

11 **Supplementary Information 2.** Raw data for the qRT-PCR experiments.

12 **ACKNOWLEDGEMENTS**

13 The authors would like to thank Maureen Liu and Sheila Keeble for assistance with
14 some research planning and laboratory work. This work was mainly funded by a
15 BBSRC DTP studentship to Harriet Johnson. The work presented here is adapted
16 from a chapter in her PhD thesis, available electronically at
17 <http://eprints.nottingham.ac.uk/33183/>

1 REFERENCES

- 2 ANDERSEN, C.L., JENSEN, J.L. & ORNTOFT, T.F. 2004 Normalization of real-time
3 quantitative reverse transcription-PCR data: A model-based variance estimation
4 approach to identify genes suited for normalization, applied to bladder and colon
5 cancer data sets. *Cancer Research*, **64**: 5245-5250.
- 6 BAVAN, S., STRAUB, V.A., WEBB, T.E. & ENNION, S.J. 2012 Cloning and
7 characterization of a P2X receptor expressed in the central nervous system of
8 *Lymnaea stagnalis*. *PLoS One*, **7**.
- 9 BOUETARD, A., BESNARD, A.L., VASSAUX, D., LAGADIC, L. & COUTELLE, C.
10 M.A. 2013 Impact of the redox-cycling herbicide diquat on transcript expression and
11 antioxidant enzymatic activities of the freshwater snail *Lymnaea stagnalis*. *Aquatic
12 Toxicology*, **126**: 256-265.
- 13 BOUETARD, A., NOIROT, C., BESNARD, A.L., BOUCHEZ, O., CHOISNE, D.,
14 ROBE, E., KLOPP, C., LAGADIC, L. & COUTELLE, C. M.A. 2012 Pyrosequencing-
15 based transcriptomic resources in the pond snail *Lymnaea stagnalis*, with a focus on
16 genes involved in molecular response to diquat-induced stress. *Ecotoxicology*, **21**:
17 2222-2234.
- 18 CARTER, C.J., RAND, C., MOHAMMAD, I., LEPP, A., VESPRINI, N., WIEBE, O.,
19 CARLONE, R. & SPENCER, G.E. 2015 Expression of a retinoic acid receptor
20 (RAR)-like protein in the embryonic and adult nervous system of a protostome
21 species. *Journal of Experimental Zoology Part B-Molecular and Developmental
22 Evolution*, **324**: 51-67.
- 23 DAVISON, A., MCDOWELL, G.S., HOLDEN, J.M., JOHNSON, H.F.,
24 KOUTSOVOULOS, G.D., LIU, M.M., HULPIAU, P., VAN ROY, F., WADE, C.M.,
25 BANERJEE, R., YANG, F., CHIBA, S., DAVEY, J.W., JACKSON, D.J., LEVIN, M. &

1 BLAXTER, M.L. 2016 Formin is associated with left-right asymmetry in the pond
2 snail and the frog. *Current Biology*, **26**: 654-660.

3 FENG, Z.P., ZHANG, Z., VAN KESTEREN, R.E., STRAUB, V.A., VAN NIEROP, P.,
4 JIN, K., NEJATBAKHSH, N., GOLDBERG, J.I., SPENCER, G.E., YEOMAN, M.S.,
5 WILDERING, W., COORSSEN, J.R., CROLL, R.P., BUCK, L.T., SYED, N.I. & SMIT,
6 A.B. 2009 Transcriptome analysis of the central nervous system of the mollusc
7 *Lymnaea stagnalis*. *BMC Genomics*, **10**: 15.

8 FOSTER, N.L., LUKOWIAK, K. & HENRY, T.B. 2015 Time-related expression
9 profiles for heat shock protein gene transcripts (HSP40, HSP70) in the central
10 nervous system of *Lymnaea stagnalis* exposed to thermal stress. *Communicative &*
11 *integrative biology*, **8**: e1040954.

12 HATAKEYAMA, D., OKUTA, A., OTSUKA, E., LUKOWIAK, K. & ITO, E. 2013
13 Consolidation of long-term memory by insulin in *Lymnaea* is not brought about by
14 changing the number of insulin receptors. *Communicative & integrative biology*, **6**:
15 e23955.

16 HELLEMANS, J., MORTIER, G., DE PAEPE, A., SPELEMAN, F. &
17 VANDESOMPELE, J. 2007 qBase relative quantification framework and software for
18 management and automated analysis of real-time quantitative PCR data. *Genome*
19 *Biol*, **8**.

20 HIBBELER, S., SCHARSACK, J.P. & BECKER, S. 2008 Housekeeping genes for
21 quantitative expression studies in the three-spined stickleback *Gasterosteus*
22 *aculeatus*. *BMC Molecular Biology*, **9**: 18.

23 HOHAGEN, J. & JACKSON, D.J. 2013 An ancient process in a modern mollusc:
24 early development of the shell in *Lymnaea stagnalis*. *BMC Developmental Biology*,
25 **13**.

1 JOHNSON, H.F. 2016 Left-right asymmetry variation in the pond snail, *Lymnaea*
2 *stagnalis*: exploring patterns of gene expression. PhD thesis, School of Life
3 Sciences, University of Nottingham.

4 KAROUSIS, E.D., NASIF, S. & MÜHLEMANN, O. 2016 Nonsense-mediated mRNA
5 decay: novel mechanistic insights and biological impact. *Wiley Interdisciplinary*
6 *Reviews: RNA*, **7**: 661-682.

7 KURODA, R., ENDO, B., ABE, M. & SHIMIZU, M. 2009 Chiral blastomere
8 arrangement dictates zygotic left-right asymmetry pathway in snails. *Nature*, **462**:
9 790-794.

10 LI, T., DIAO, H., ZHAO, L., XING, Y., ZHANG, J., LIU, N., YAN, Y., TIAN, X., SUN,
11 W. & LIU, B. 2017 Identification of suitable reference genes for real-time quantitative
12 PCR analysis of hydrogen peroxide-treated human umbilical vein endothelial cells.
13 *BMC Molecular Biology*, **18**: 10.

14 LIU, M.M., DAVEY, J.W., JACKSON, D.J., BLAXTER, M.L. & DAVISON, A. 2014 A
15 conserved set of maternal genes? Insights from a molluscan transcriptome.
16 *International Journal of Developmental Biology*, **58**: 501-511.

17 LU, T.Z. & FENG, Z.-P. 2011 A sodium leak current regulates pacemaker activity of
18 adult central pattern generator neurons in *Lymnaea stagnalis*. *PLoS One*, **6**: e18745.

19 LYKKE-ANDERSEN, S. & JENSEN, T.H. 2015 Nonsense-mediated mRNA decay:
20 an intricate machinery that shapes transcriptomes. *Nature Reviews Molecular Cell*
21 *Biology*, **16**: 665-677.

22 OLIAS, P., ADAM, I., MEYER, A., SCHARFF, C. & GRUBER, A.D. 2014 Reference
23 genes for quantitative gene expression studies in multiple avian species. *PLoS One*,
24 **9**: e99678.

1 PFAFFL, M.W. 2001 A new mathematical model for relative quantification in real-
2 time RT-PCR. *Nucleic Acids Research*, **29**.

3 PFAFFL, M.W., TICHOPAD, A., PRGOMET, C. & NEUVIANS, T.P. 2004
4 Determination of stable housekeeping genes, differentially regulated target genes
5 and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations.
6 *Biotechnology Letters*, **26**.

7 RADONIC, A., THULKE, S., MACKAY, I.M., LANDT, O., SIEGERT, W. & NITSCHKE,
8 A. 2004 Guideline to reference gene selection for quantitative real-time PCR.
9 *Biochemical and Biophysical Research Communications*, **313**: 856-862.

10 RIBEIRO, M., SCHOFIELD, M., KEMENES, I., BENJAMIN, P.R., O'SHEA, M. &
11 KORNEEV, S.A. 2010 Atypical guanylyl cyclase from the pond snail *Lymnaea*
12 *stagnalis*: cloning, sequence analysis and characterization of expression.
13 *Neuroscience*, **165**: 794-800.

14 ROZEN, S. & SKALETSKY, H.J. 2006 Primer3 on the WWW for general users and
15 for biologist programmers. In: *Bioinformatics Methods and Protocols*: (Krawetz, S.,
16 Misener, S. and Totowa, N.J., eds). Methods in Molecular Biology, Humana Press.

17 SHIBAZAKI, Y., SHIMIZU, M. & KURODA, R. 2004 Body handedness is directed by
18 genetically determined cytoskeletal dynamics in the early embryo. *Current Biology*,
19 **14**: 1462-1467.

20 SIRAKOV, M., ZARRELLA, I., BORRA, M., RIZZO, F., BIFFALI, E., ARNONE, M.I. &
21 FIORITO, G. 2009 Selection and validation of a set of reliable reference genes for
22 quantitative RT-PCR studies in the brain of the cephalopod mollusc *Octopus*
23 *vulgaris*. *BMC Molecular Biology*, **10**: 70.

24 TEE, Y.H., SHEMESH, T., THIAGARAJAN, V., HARIADI, R.F., ANDERSON, K.L.,
25 PAGE, C., VOLKMANN, N., HANEIN, D., SIVARAMAKRISHNAN, S., KOZLOV,

1 M.M. & BERSHADSKY, A.D. 2015 Cellular chirality arising from the self-organization
2 of the actin cytoskeleton. *Nature Cell Biology*, **17**: 445-457.

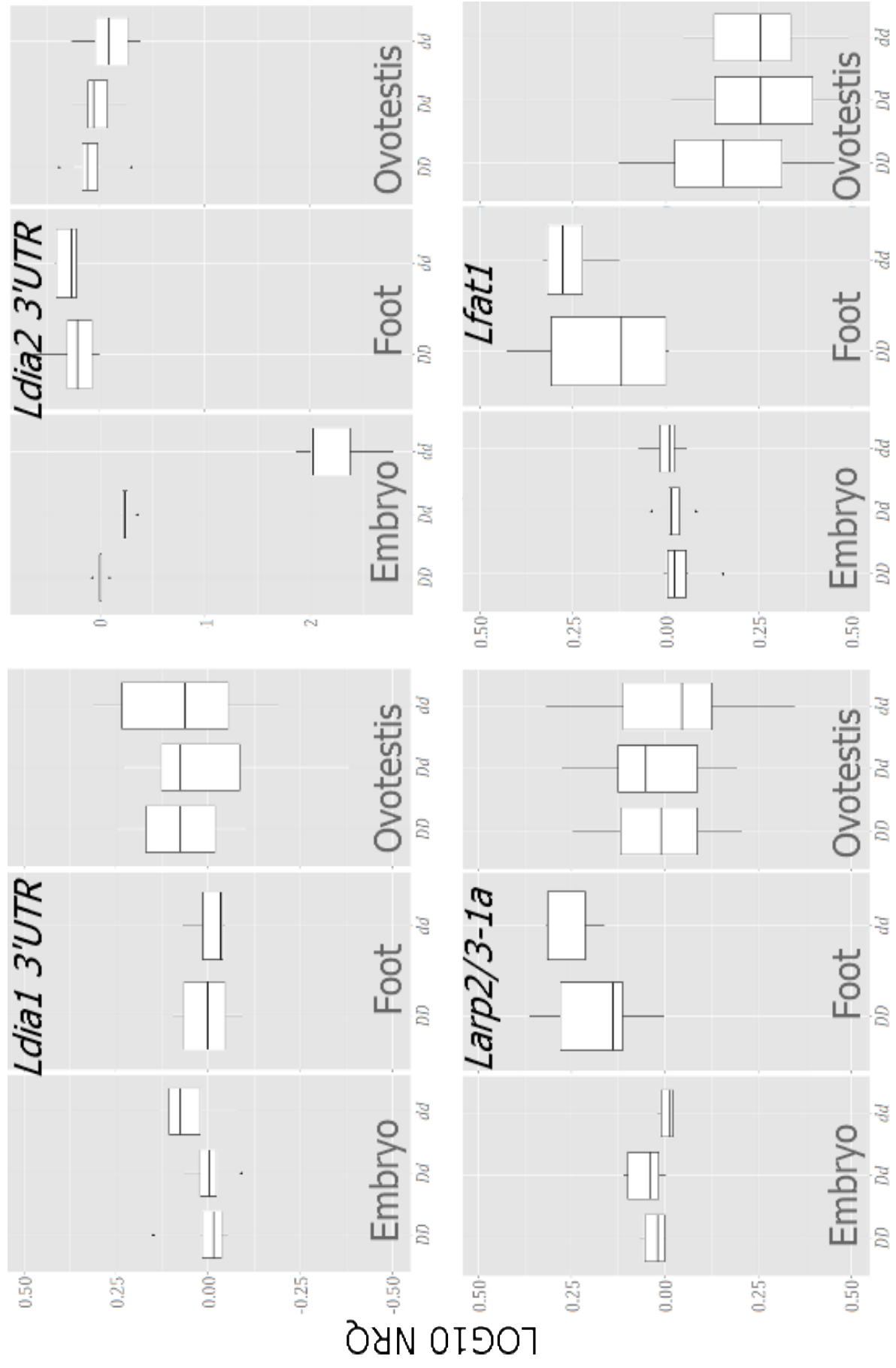
3 VAN KESTEREN, R.E., CARTER, C., DISSEL, H.M.G., VAN MINNEN, J.,
4 GOUWENBERG, Y., SYED, N.I., SPENCER, G.E. & SMIT, A.B. 2006 Local
5 synthesis of actin-binding protein beta-thymosin regulates neurite outgrowth. *Journal*
6 *of Neuroscience*, **26**: 152-157.

7 VAN NIEROP, P., BERTRAND, S., MUNNO, D.W., GOUWENBERG, Y., VAN
8 MINNEN, J., SPAFFORD, J.D., SYED, N.I., BERTRAND, D. & SMIT, A.B. 2006
9 Identification and functional expression of a family of nicotinic acetylcholine receptor
10 subunits in the central nervous system of the mollusc *Lymnaea stagnalis*. *Journal of*
11 *Biological Chemistry*, **281**: 1680-1691.

12 VANDESOMPELE, J., DE PRETER, K., PATTYN, F., POPPE, B., VAN ROY, N., DE
13 PAEPE, A. & SPELEMAN, F. 2002 Accurate normalization of real-time quantitative
14 RT-PCR data by geometric averaging of multiple internal control genes. *Genome*
15 *Biology*, **3**.

16

Figure 1. Boxplots showing Log scale NRQ values (LOG10 NRQ) for four different genes in three genotypes, *DD*, *Dd* and *dd* across three tissues, embryo, foot and ovotestis tissue, relative to a single standard *DD* snail. *Ldia2 3'UTR* transcripts are almost absent from the embryo in *dd* individuals, and also show reduced expression in the ovotestis. This effect is not seen in the foot tissue. The graphs also show that, in general, between-sample variation is least in the embryo.



Genotype

Figure 2. Boxplots showing Log scale NRQ values (LOG10 NRQ) for six different genes in three different tissue), using a single genotype *DD*, and relative to a single standard. The expression of five genes is depleted in the embryo, with the exception of *Ldia2*.

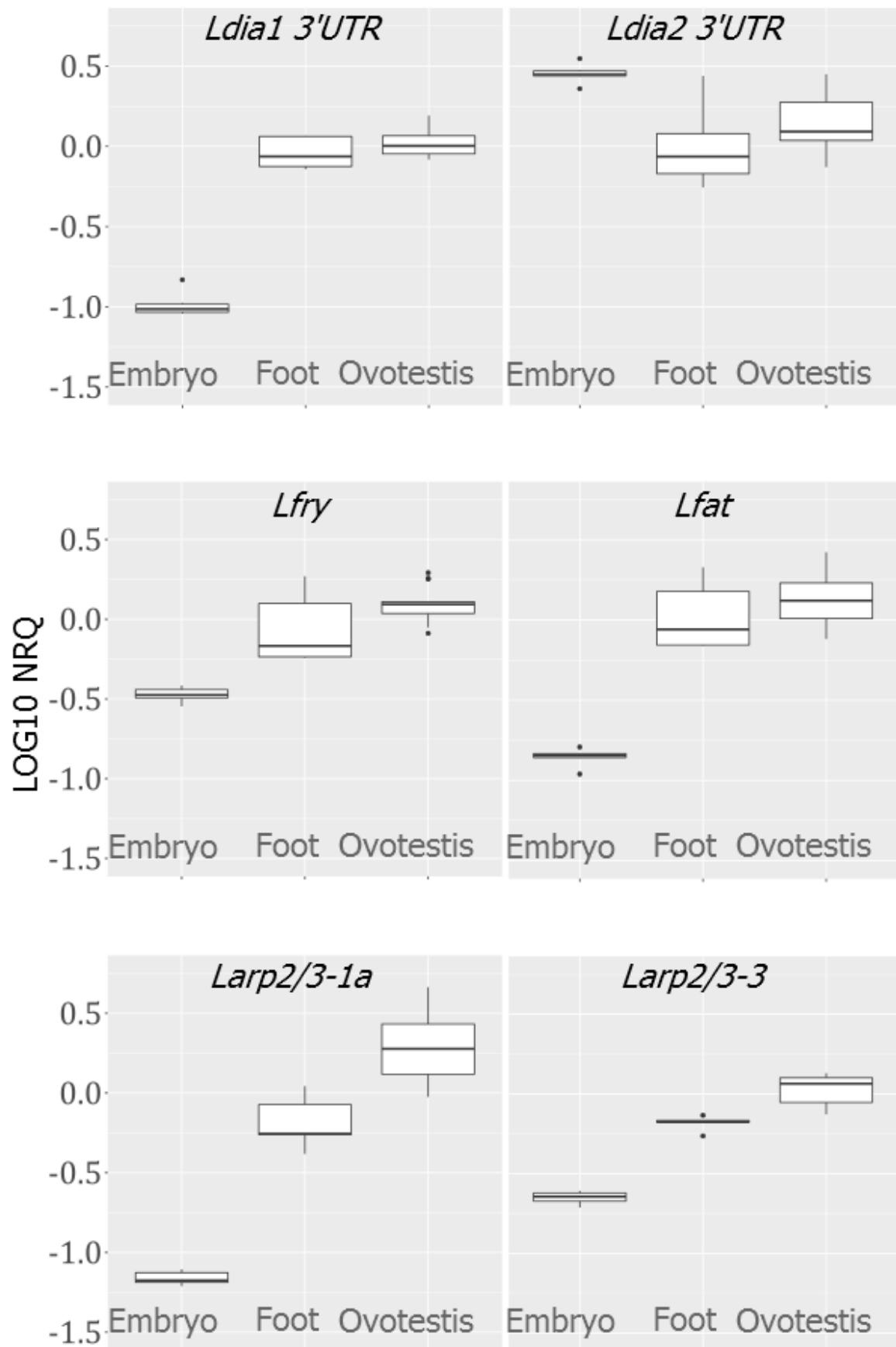


Table 1. Primers used for the amplification of endogenous control genes (top) and the tested cytoskeletal genes (bottom), including the estimated intron size and the minimum concentration of sample cDNA (as a percentage of full concentration) required to achieve the amplification efficiency. * indicates primer crosses exon/intron boundary.

ID	Endogenous controls	Primers	Tm (°C)	Intron (bp)	Efficiency	Min cDNA conc (%)
<i>Lacads</i>	acyl-CoA dehydrogenase	TGCACTCTCTAAACGAACCTCC	58.4	866	1.912	0.27
		TCCCTTGATTGTGCTGTTGAC	58.8			
<i>Lef1</i>	elongation factor 1-alpha	CGTCACAACCAGCATATCCC	58.7	663	2.115	0.67
		AGAGTTCGAGGGCTGCTTAC*	59.5			
<i>Lhis2a</i>	histone H2A	TCAGAGGAGATGAGGAGTTGG	58.3	785†	1.943	0.03
		CCCCAAGTTATGCTGCCTTC	58.9			
<i>Lrpl14</i>	60S ribosomal protein L14	TAATAAGTCGGTTGCGCGC*	59.0	2254	1.906	0.03
		GGGAACAGTCTACTTGGGC*	57.5			
<i>Lube2</i>	ubiquitin-conjugating E2	GCGGATCCTCTTGAATCTT*	58.3	3224	1.923	0.03
		TCTGTGGACTGCATATCACTCT	58.6			
<i>Lywhaz</i>	14-3-3 protein zeta	GGAGGAGCTGAAGTCAATATGC	58.9	711	1.918	0.03
		AGTCACCCTGCATTTTGAGG	58.1			
ID	Cytoskeletal genes	Primers	Tm (°C)	Intron (bp)	Efficiency	Min cDNA conc (%)
Ldia1 3'UTR	diaphanous-related formin	AGTGGTGTGGGCAAAAGATG	58.7	n/a	1.986	0.27
		TATTCTGTTGATGCACGGCC	58.6			
Ldia2 3'UTR	diaphanous-related formin	GGGAGTTCAAGTTCAAGCCTATC	59.1	n/a	1.912	0.27
		GGCAAGCTACGACTCTTCTC	58.1			
Ldia2 ORF	diaphanous-related formin	GGGTGACAATGAAGTGGACC	58.5	n/a	1.948	1.33
		ACATGCATCTGTAACATCTGCC	59.1			
Lfry	furry	ACTTACCCTGCTCAAATGCC	58.2	717	1.876	0.59
		ATGTTTCTGTGCTGCCGTC	59.4			
Lfat	fat-like cadherin	TGCCCATGTTGCTAAGTTCAG	58.8	1347	1.838	0.59
		CCTCTATCCCAGTTCGACGG	59.9			
Larp2/3-1a	actin-related protein 1a	CTGAAAATAGCCTTGTTCAGC	58.8	343	1.847	0.67
		CCAGACTCCTTTCTGGGAC	60.0			
Larp2/3-3	actin-related protein 3	AGCCAGCTAACAAGGAGAAG	59.7	520	1.775	0.67
		AGCATAGCCACCATTGCTTG*	59.5			

Table 2. Summary of recommended single genes and gene pairs to use for each tissue and according to the normalising control software method. Although there is little consistency in the top genes to use across tissues and methods, in reality, all six endogenous control genes are acceptable for use.

Tissue	GeNorm	NormFinder	BestKeeper, SD
Embryo	<i>Lhis2a/Lube2</i>	<i>Lhis2a</i> or <i>Lacads/Lube2</i>	<i>Lhis2a/Lywhaz</i>
Foot	<i>Lyhwaz/Lube2</i>	<i>Lywhaz</i> or <i>Lywhaz/Lube2</i>	<i>Lrpl14/Lacads</i>
Ovotestis	<i>Lrpl14/Lube2</i>	<i>Lhis2a</i> or <i>Lef1/Lywhaz</i>	<i>Lrpl14/Lube2</i>

Table 3. Gene expression stability results per tissue, using three different normalising control methods. geNorm provides the best paired combination of genes, with additional V scores indicating the best accumulative combination and individual M scores giving a measure of individual expression stability. Normfinder provides the best combined pair of genes with a separate associated stability score. Bestkeeper results are presented as both their correlation with the BestKeeper index (r), with associated probability values (P), and the standard deviation (SD) associated with the average Cq per gene.

Tissue	geNorm			NormFinder			BestKeeper, SD			BestKeeper, r		
	Target	Stability score	V score	M score	Target	Stability score	Stability Best Pair	Stability Value	Target	SD	Target	r
Embryo, n=12	<i>Lhis2a/</i>	0.196	0.061	0.246/	<i>Lhis2a</i>	0.058	<i>Lacads/</i>	<i>Lhis2a</i>	0.408	<i>Lhis2a</i>	0.979	0.001
	<i>Lube2</i>			0.259	<i>Lrp14</i>	0.076	<i>Lube2</i>	<i>Lywhaz</i>	0.457	<i>Lef1a</i>	0.969	0.001
	<i>Lrp14</i>	0.204	0.064	0.267	<i>Lube2</i>	0.086		<i>Lrp14</i>	0.476	<i>Lube2</i>	0.962	0.001
	<i>Lacads</i>	0.242	0.052	0.282	<i>Lacads</i>	0.104		<i>Lacads</i>	0.493	<i>Lrp14</i>	0.957	0.001
	<i>Lywhaz</i>	0.262	0.049	0.324	<i>Lef1a</i>	0.122		<i>Lube2</i>	0.514	<i>Lacads</i>	0.949	0.001
	<i>Lef1a</i>	0.285	n/a	0.330	<i>Lywhaz</i>	0.124		<i>Lef1a</i>	0.577	<i>Lywhaz</i>	0.900	0.001
	<i>Lywhaz/</i>	0.217	0.092	0.325/	<i>Lywhaz</i>	0.074	<i>Lube2/</i>	<i>Lrp14</i>	0.500	<i>Lywhaz</i>	0.998	0.001
	<i>Lube2</i>			0.401	<i>Lube2</i>	0.133	<i>Lywhaz</i>	<i>Lacads</i>	0.638	<i>Lube2</i>	0.993	0.001
	<i>Lhis2a</i>	0.269	0.091	0.461	<i>Lacads</i>	0.151		<i>Lef1a</i>	0.695	<i>Lhis2a</i>	0.984	0.001
	<i>Lacads</i>	0.327	0.082	0.407	<i>Lrp14</i>	0.176		<i>Lywhaz</i>	0.754	<i>Lacads</i>	0.981	0.001
	<i>Lrp14</i>	0.376	0.088	0.489	<i>Lhis2a</i>	0.215		<i>Lube2</i>	0.867	<i>Lrp14</i>	0.964	0.001
	<i>Lef1a</i>	0.444	n/a	0.579	<i>Lef1a</i>	0.298		<i>Lhis2a</i>	0.947	<i>Lef1a</i>	0.907	0.001
Ovotestis, n=9	<i>Lrp14/</i>	0.250	0.097	0.367/	<i>Lhis2a</i>	0.124	<i>Lef1a/</i>	<i>Lrp14</i>	0.176	<i>Lywhaz</i>	0.894	0.001
	<i>Lube2</i>			0.363	<i>Lrp14</i>	0.147	<i>Lywhaz</i>	<i>Lube2</i>	0.313	<i>Lube2</i>	0.877	0.002
	<i>Lywhaz</i>	0.292	0.070	0.384	<i>Lube2</i>	0.153		<i>Lhis2a</i>	0.330	<i>Lacads</i>	0.876	0.002
	<i>Lhis2a</i>	0.309	0.079	0.360	<i>Lywhaz</i>	0.171		<i>Lywhaz</i>	0.366	<i>Lrp14</i>	0.853	0.003
<i>Lacads</i>	0.360	0.077	0.473	<i>Lacads</i>	0.206		<i>Lef1a</i>	0.369	<i>Lhis2a</i>	0.831	0.005	
<i>Lef1a</i>	0.409	n/a	0.507	<i>Lef1a</i>	0.243		<i>Lacads</i>	0.500	<i>Lef1a</i>	0.655	0.056	

Table 4. Normalised relative quantities (NRQ) of each gene, presented as a geometric mean per genotypic group (Geno), relative to different genotypes.

Heterozygote snails, *Dd*, were not used with foot tissue.

	Genotype	N	<i>Larp2/3-1a</i>	<i>Larp2/3-3</i>	<i>Ldia1 3'UTR</i>	<i>Ldia2 3'UTR</i>	<i>Ldia2 ORF</i>	<i>Lfat</i>	<i>Lfry</i>
Embryo	<i>DD</i>	6	1	1	1	1	1	1	1
	<i>Dd</i>	5	1.059	0.735	0.969	0.563	0.476	1.052	0.984
	<i>dd</i>	6	0.926	0.960	1.111	0.006	0.029	1.103	0.960
Foot	<i>DD</i>	5	1	1	1	1	1	1	1
	<i>dd</i>	5	1.218	1.053	0.973	1.154	1.425	1.217	1.041
Ovotestis	<i>DD</i>	14	1	1	1	1	1	1	1
	<i>Dd</i>	8	1.087	0.758	0.843	0.800	0.809	0.832	0.910
	<i>dd</i>	14	0.965	0.877	1.017	0.619	0.686	0.847	0.878

Table 5. Wilcoxon rank test results for pairwise comparisons between genotypes *DD*, *Dd* and *dd* within embryo, foot and ovotestis tissue for cytoskeletal genes. The Wilcoxon rank value (*W*) is presented with the associated probability value (*P*).

Statistical significance is highlighted via * <0.05, ** <0.01.

Gene	Tissue	<i>DD</i> versus <i>dd</i>				<i>DD</i> versus <i>Dd</i>				<i>Dd</i> versus <i>dd</i>						
		<i>N, DD</i>	<i>N, dd</i>	<i>W</i>	<i>P</i>	<i>N, DD</i>	<i>N, Dd</i>	<i>W</i>	<i>P</i>	<i>N, Dd</i>	<i>N, dd</i>	<i>W</i>	<i>P</i>			
<i>Larp2/3-1a</i>	Embryo	6	6	30	0.065	6	5	10	0.429	5	6	27	0.03	*		
	Foot	5	5	7	0.31	n/a										
	Ovotestis	14	14	106	0.735	14	8	50	0.714	8	14	65	0.57			
<i>Larp2/3-3</i>	Embryo	6	6	23	0.485	6	5	29	0.009	**	5	6	3	0.03	*	
	Foot	5	5	6	0.222	n/a										
	Ovotestis	14	14	117	0.401	14	8	81	0.095	8	14	50	0.714			
<i>Ldia1 3'UTR</i>	Embryo	6	6	12	0.394	6	5	15	1	5	6	7	0.178			
	Foot	5	5	13	1	n/a										
	Ovotestis	14	14	95	0.91	14	8	61	0.764	8	14	47	0.57			
<i>Ldia2 3'UTR</i>	Embryo	6	6	36	0.002	**	6	5	30	0.004	**	5	6	30	0.004	**
	Foot	5	5	8	0.421	n/a										
	Ovotestis	14	14	155	0.008	**	14	8	73	0.267	8	14	74	0.238		
<i>Ldia2 ORF</i>	Embryo	6	6	36	0.002	**	6	5	30	0.004	**	5	6	30	0.004	**
	Foot	5	5	7	0.31	n/a										
	Ovotestis	14	14	152	0.012	*	14	8	74	0.238	8	14	68	0.441		
<i>Lfat</i>	Embryo	6	6	11	0.31	6	5	14	0.931	5	6	12	0.662			
	Foot	5	5	8	0.421	n/a										
	Ovotestis	14	14	123	0.265	14	8	70	0.365	8	14	53	0.868			
<i>Lfry</i>	Embryo	6	6	25	0.31	6	5	16	0.931	5	6	19	0.537			
	Foot	5	5	11	0.841	n/a										
	Ovotestis	14	14	112	0.541	14	8	69	0.402	8	14	56	1			

Table 6. Normalised relative quantities (NRQ) of each gene, not log transformed, presented as a geometric mean per genotypic group and tissue, relative to the same single reference standard. Heterozygote snails, *Dd*, were not used with foot tissue.

Tissue	Genotype	N	<i>Larp2/3-1a</i>	<i>Larp2/3-3</i>	<i>Ldia1 3'UTR</i>	<i>Ldia2 3'UTR</i>	<i>Ldia2 ORF</i>	<i>Lfat</i>	<i>Lfry</i>
Embryo	<i>DD</i>	6	0.069	0.224	0.103	2.835	1.973	0.14	0.336
	<i>Dd</i>	5	0.077	0.175	0.105	1.676	0.988	0.154	0.348
	<i>dd</i>	6	0.065	0.219	0.118	0.019	0.058	0.158	0.33
Foot	<i>DD</i>	5	0.652	0.656	0.91	1.012	0.708	1.062	0.88
	<i>dd</i>	5	0.784	0.682	0.875	1.153	0.997	1.277	0.905
Ovotestis	<i>DD</i>	14	1.935	1.068	1.049	1.418	1.553	1.339	1.239
	<i>Dd</i>	8	2.256	0.869	0.95	1.218	1.348	1.196	1.21
	<i>dd</i>	14	1.82	0.914	1.04	0.855	1.039	1.105	1.06