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**Reference gene selection and RNA preservation protocol in the cat flea, *Ctenocephalides felis*, for gene expression studies.**

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2 *Ctenocephalides felis*, for gene expression studies.

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## 29 SUMMARY

30 The cat flea, *Ctenocephalides felis*, is a major pest species on companion animals thus of  
31 significant importance to the animal health industry. The aim of this study was to develop  
32 sampling and storage protocols and identify stable reference genes for gene expression  
33 studies to fully utilise the growing body of molecular knowledge of *C. felis*. RNA integrity  
34 was assessed in adult and larvae samples, which were either pierced or not pierced and stored  
35 in RNAlater at ambient temperature. RNA quality was maintained best in pierced samples,  
36 with negligible degradation evident after 10 days. RNA quality from non-pierced samples  
37 was poor within 3 days. Ten candidate reference genes were evaluated for their stability  
38 across four group comparisons (developmental stages, genders, feeding statuses and  
39 insecticide-treatment statuses). Glyceraldehyde 3 phosphate dehydrogenase (GAPDH), 60S  
40 ribosomal protein L19 (RPL19) and elongation factor-1 $\alpha$  (Ef) were ranked highly in all  
41 stability comparisons, thus are recommended as reference genes under similar conditions.  
42 Employing just two of these three stable reference genes was sufficient for accurate  
43 normalization. Our results make a significant contribution to the future of gene expression  
44 studies in *C. felis*, describing validated sample preparation procedures and reference genes for  
45 use in this common pest.

46

47 Key words: *Ctenocephalides felis*, quantitative real-time PCR, normalization, RNA, gene  
48 expression, cat flea, RNA quality, RNA degradation

49

## 50 KEY FINDINGS

- 51 • Piercing *C. felis* samples in RNA-stabilizing solutions is critical to maintain RNA  
52 quality
- 53 • GAPDH, Elongation factor 1 $\alpha$  and RPL19 are the most stable reference genes in *C.*  
54 *felis*
- 55 • The above three genes are stable across life stages, feeding status and insecticide  
56 treatment
- 57 • Employing just two of these three stable reference genes was sufficient for accurate  
58 normalization.
- 59 • Case study of vitellogenin expression demonstrates necessity to use multiple reference  
60 genes
- 61

## 62 INTRODUCTION

63 The cat flea, *Ctenocephalides felis*, is one of the most common ectoparasites infesting  
64 companion animals worldwide and is of major importance to pet owners and the animal  
65 health industry (Rust and Dryden 1997; Beugnet et al. 2014). As well as irritation, cat flea  
66 infestations can trigger a severe allergic reaction in companion animals, known as flea allergy  
67 dermatitis (FAD), and act as a vector for several bacterial infections, most notably *Rickettsia*  
68 *felis*, and the parasitic worm *Dipylidium caninum* (Traversa 2013). For these reasons, and  
69 also the potential for current treatments to become ineffective, there is a constant need for  
70 more insight into this species. In recent years several cat flea expressed sequence tag (EST)  
71 and transcriptome studies have become available (Gaines et al. 2002; Ribeiro et al. 2012;  
72 Misof et al. 2014; Green et al. 2015), adding to a growing body of molecular knowledge that  
73 opens new opportunities for control. Techniques such as reverse-transcription quantitative  
74 real-time PCR (RT-qPCR) can be used to explore gene expression and this information can  
75 be used to find new ways to control *C. felis*.

76 RT-qPCR allows precise measurement of differential gene expression between samples. The  
77 sensitivity of the technique makes detection of small changes possible; however it also makes  
78 the results susceptible to the introduction of errors from experimental technique, such as  
79 differences in initial sample size, RNA extraction efficacy and reverse transcriptase enzyme  
80 efficiency during cDNA synthesis. To correct for these errors normalization is performed.  
81 Several normalization strategies can be used, such as accounting for the amount of total  
82 RNA, standardising sample size, or utilising internal reference genes, which are subject to  
83 conditions similar to the mRNA of interest (Huggett et al. 2005). Use of one or more  
84 endogenous reference genes has emerged as the preferred method for relative quantification  
85 and because they undergo the same processes as the mRNA of interest, reference genes can  
86 be used to correct for experimentally-introduced differences between samples (Derveaux et  
87 al. 2010). An ideal reference gene would be stably expressed across all experimental groups.

88 While normalization using endogenous reference genes is common, it is often the case that  
89 such reference genes are chosen without proper validation. Traditional “housekeeping” genes,  
90 such as  $\beta$ -Actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), have frequently  
91 been used as reference genes for RT-qPCR without proper assessment of their suitability,  
92 largely due to their historic use as controls in less sensitive quantitative approaches such as  
93 Northern blotting (Boda et al. 2008). When tested, many commonly used control genes have

94 been shown to exhibit unstable expression across treatments under various conditions  
95 (Thellin et al. 1999). Several studies demonstrating the impact of unstable reference genes on  
96 the assessment of target gene expression levels have highlighted the need to validate  
97 reference genes for specific experimental design, cell and tissue type (Kidd et al. 2007; Boda  
98 et al. 2008; Kosir et al. 2010).

99 The aim of the current study was to develop procedures and tools for working with cat flea  
100 specimens at a molecular level. Understanding how storage can impact RNA integrity is vital  
101 for implementation of collaboration between research centres, allowing the transfer of  
102 reliable RNA between groups. Reliable reference genes are essential for robust gene  
103 expression studies (Bustin et al. 2009). Therefore the main tasks were to investigate how  
104 sample collection and storage procedures affect integrity of RNA that will be used in  
105 downstream gene expression studies and to screen and validate reference genes for use in RT-  
106 qPCR screens in the cat flea. Ten candidate reference genes in *C. felis* were assessed across  
107 the following 4 groups: developmental stage, sex, feeding status (fed versus unfed) and  
108 insecticide treatment-status (treated or untreated).

## 109 MATERIALS AND METHODS

### 110 *Insect rearing*

111 All *C. felis* samples were obtained from an artificially reared colony kept by Zoetis Inc  
112 (Kalamazoo, MI, USA), developed from fleas supplied by Elward II, California, USA, using  
113 methods similar to Kernif et al. (2015). Adults were fed *ad libitum* on bovine blood, after  
114 which eggs were collected three times per week and placed in containers with larval rearing  
115 media, consisting of 74 % finely ground laboratory canine diet, 25 % dried Brewer's yeast  
116 and 1 % part dried bovine blood, and fine sand. Larval containers were left undisturbed until  
117 emergence of adults approximately three weeks after egg collection. All life stages were  
118 reared in an insectary at  $\approx 26$  °C and 80 % relative humidity with a 12:12 L:D cycle.

### 119 *Biological samples and cDNA synthesis*

120 Fed adult *C. felis* of mixed ages were collected from adult feeding chambers. Larvae and  
121 pupae were collected from culture pots approximately 7 and 12 days post-hatch, respectively.  
122 Unfed adults were collected approximately 30 days post-hatch (within 3 days of emergence  
123 from pupal case). For insecticide treatment, adults of mixed age were allowed to feed on 1

124  $\mu\text{M}$  selamectin (Zoetis Inc, USA) in bovine blood for 24 hours prior to collection. Cat flea  
125 samples were pierced once, centrally, with a 23 gauge needle, and groups of 10 placed  
126 directly in 1 mL RNAlater (Life Technologies, ThermoFisher Scientific, Grand Island, NY,  
127 USA) and kept at 4 °C overnight before storage at -80°C. Samples were sent to the University  
128 of Aberdeen, UK, on dry ice. Prior to RNA extraction, pupae were removed from their cases  
129 using 23 gauge needles. On the basis of size, females being larger than males, a subset of fed  
130 adults were sorted into males and females.

131 For RNA extraction, pools of 3-10 fleas were removed from RNAlater and then homogenised  
132 in 1 mL Tri-reagent (Sigma-Aldrich, UK) by crushing in 1.5 mL microfuge tubes with  
133 micropestles. RNA was extracted according to manufacturer's instructions, with the phase  
134 separation and ethanol washes repeated twice. RNA was resuspended in 8  $\mu\text{L}$  (selamectin-  
135 treated samples, as fewer fleas were available for RNA extraction) or 20  $\mu\text{L}$  RNase-free  $\text{H}_2\text{O}$   
136 and quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific,  
137 Loughborough, UK). RNA was treated with RQ1 DNase (Promega, UK) and 1  $\mu\text{g}$  used as  
138 template for cDNA synthesis with BioScript reverse transcriptase (Bioline Reagents Limited,  
139 London, UK).

#### 140 *Assessing influence of sampling procedure and storage conditions on RNA integrity*

141 Groups of 10 larvae and fed adults were either pierced once with a 23 gauge needle or not  
142 pierced and placed in 1 mL RNAlater (Life Technologies). All samples were incubated at 4  
143 °C overnight then stored at room temperature for 0, 3 or 10 days before being frozen at -80  
144 °C until processing. RNA was extracted from groups of 10 fleas, as above. Total RNA  
145 concentration was measured using a ND-1000 Nanodrop spectrophotometer (Thermo-  
146 scientific) and RNA quality was assessed using an Agilent 2100 Bioanalyzer and Agilent  
147 RNA 6000 Nano kit. Due to a hidden 18S/28S break in the rRNA of many arthropod species  
148 (also apparent in *C. felis*) an accurate RNA Integrity Number (RIN) cannot be calculated  
149 (Winnebeck et al. 2010). RNA integrity was therefore assessed by visual inspection of  
150 electropherograms for each sample, assessing two replicates for each treatment. The time  
151 points of 3 and 10 days were selected for study relates to the approximate time for  
152 international courier by air (3 days) and international surface mail (10 days).

153 *Candidate reference gene selection & primer design*

154 Ten reference gene candidates were selected based on housekeeping genes previously used  
155 for RT-qPCR in the cat flea (Dreher-Lesnack et al. 2010) or transcripts commonly used as  
156 references in other insect species (Scharlaken et al. 2008; Li et al. 2013; Zhai et al. 2014; Tan  
157 et al. 2015). Ten candidate primer sets, representing transcripts from different functional  
158 classes, were initially assessed (Table 1). Sequences were obtained from annotated sequences  
159 in Ribeiro et al. (2012) (18S ribosomal RNA (18S), 28S ribosomal RNA (28S), 60S  
160 ribosomal protein (RPL19)), the BLAST Transcriptome Shotgun Assembly database  
161 (Elongation factor 1  $\alpha$  (Ef), Act ( $\beta$ -Actin)), or by using tBLASTn to search the cat flea EST  
162 database using *Drosophila melanogaster* sequences obtained from Flybase (Dos Santos et al.  
163 2015) (GAPDH, Heat shock protein 22 (HSP22), NADH dehydrogenase/ ubiquinone  
164 reductase (NADH),  $\alpha$ -Tubulin ( $\alpha$ Tub)). Primer3Plus ([http://www.bioinformatics.nl/cgi-  
165 bin/primer3plus/primer3plus.cgi/](http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/)) was used for primer design, implementing the qPCR  
166 setting and then checked manually. For comparison, a primer set targeting muscle-specific  
167 actin (DLAct) used in Dreher-Lesnack et al. (2010) was also included in the analysis. PCR  
168 was performed for each primer set using 25  $\mu$ L BioMix Red (Bioline), 22  $\mu$ L H<sub>2</sub>O, 2  $\mu$ L  
169 mixed *C. felis* cDNA and 1  $\mu$ L 10 mM primer sets. Reactions were performed with the  
170 following conditions: 95 °C 5 min, 35 cycles of 95 °C 30 s, 58 °C 45 s, 72 °C 45 s, followed  
171 by incubation at 72 °C for 10 min. PCR products were electrophoresed in a 2 % agarose Tris-  
172 borate-EDTA (TBE) gel to confirm there was a single product of the expected size.

173 *Quantitative real-time PCR*

174 RT-qPCR was carried out in 96-well plates CFX96 Touch Real-Time PCR detection system  
175 (Bio-Rad Laboratories, USA). Reactions were run in 20  $\mu$ L volumes (10  $\mu$ L iTaq Sybr Green  
176 supermix (Bio-Rad), 1  $\mu$ L 10 mM primer mix, 5  $\mu$ L H<sub>2</sub>O and 4  $\mu$ L template cDNA (1/20  
177 dilution of cDNA produced from 1  $\mu$ g DNase-treated RNA). PCR cycling conditions were:  
178 95 °C 3min, 40 cycles of 95 °C 10 s, 58 °C 30 s followed by a melt-curve analysis step  
179 consisting of 0.5 °C incremental rises every 5 s, rising from 65°C to 95 °C. No template  
180 controls in duplicate were run for each primer set. Three replicates were run in triplicate for  
181 each treatment, except unfed and fed adults, where two and four replicates were used,  
182 respectively. Four-step 10-fold serial dilutions of mixed standard cDNA were performed in  
183 duplicate to create standard curves to calculate primer efficiencies. CFX manager software  
184 (version 3.1) (Biorad) was used to calculate efficiencies from a standard serial dilution curve.



185 Melt-curve analysis utilised CFX manager software to confirm correct product profiles for  
186 each primer set and Cq values extracted for further analysis.

187 *Data analysis*

188 Reference gene stability was assessed using three software programmes: geNorm (version  
189 3.4) (Vandesompele et al. 2002), Normfinder (version 0.953) (Andersen et al. 2004) and  
190 Bestkeeper (version 1.0) (Pfaffl et al. 2004). Cq values were transformed using the delta-Ct  
191 method for analysis in GeNorm. For Normfinder, Cq values were transformed to a linear  
192 scale using the calculation  $(2E)^{-Cq}$ . Cq and efficiency values were input directly into  
193 Bestkeeper.

194 GeNorm ranks reference genes from most to least stable by calculating the gene expression  
195 stability M, the average pairwise variation of the expression ratio of a particular gene  
196 compared to all other tested genes (Vandesompele et al. 2002). Low M value is indicative of  
197 gene stability, with  $M < 1.5$  necessary for utility as a reference gene. GeNorm gives two  
198 informative outputs. Firstly, a ranking of genes in order of stability based on calculation of  
199 average M for all genes and step-wise exclusion of the least stable gene and recalculation of  
200 the average M. Secondly, stability rankings generated from geNorm software can be used to  
201 assess the number of reference genes needed for accurate normalization, based on the  
202 pairwise variation ( $V_n/V_{n+1}$ ) between sequential normalization factors, based on geometric  
203 means of the most stable genes which is recalculated following addition of each subsequent  
204 gene. The lowest number of genes giving  $V_n/V_{n+1} < 0.15$  is the minimal number that should  
205 be used for normalization.

206 Normfinder utilises a model-based approach to assess reference gene stability, based on  
207 measures on intra- and inter-group variations, which are based on user-specified groupings  
208 (Andersen et al. 2004). This generates a stability value (SV) for each gene, as well as for the  
209 best combination of two reference genes. Low SV is indicative of gene stability, with  $SV > 1$   
210 suggesting a candidate is unstable and not suitable for use as a reference gene.

211 Bestkeeper uses input Cq and efficiency data to generate descriptive statistics for each gene,  
212 before generating a Bestkeeper index value ( $r$ ) for each sample based on the geometric mean  
213 of its Cq values for each reference gene tested (Pfaffl et al. 2004). Stability can be assessed,  
214 based on standard deviation ( $SD$ )  $\pm$  Cq and coefficient of variation. Only candidates where  
215  $SD \pm Cq$  is  $< 1$  are suitable for use as reference genes.

216 Analysis was conducted in each of the programmes to assess reference genes most suitable  
217 for use in four groups: Developmental stages (larvae vs pupae vs unfed adults vs fed adults),  
218 Sexes (male vs female fed adults), Feeding statuses (fed vs unfed adults) and Treatment  
219 statuses (selamectin treated vs untreated fed adults). An overall ranking was produced using a  
220 points-based system to combine the rankings from all of the programmes used.

#### 221 *Validation of reference genes – a case study in vitellogenin C expression*

222 Vitellogenins are key components of yolk in insect, produced in the fat body of adult females  
223 (Pan et al. 1969). Due to this function it is expected that levels of vitellogenin transcripts will  
224 be significantly higher in females than in males. The expected large difference made this a  
225 promising target to validate candidate reference genes for their utility in normalization.  
226 Primers were designed from an EST sequence representing vitellogenin C (Ribeiro et al.  
227 2012), tested for specificity by melt-curve analysis and PCR followed by gel electrophoresis  
228 to confirm a single product of the expected size was produced (Table 1). The efficiency of  
229 this primer set was assessed by creating a standard curve using CFX Manager software  
230 (version 3.1) (Biorad) from duplicate 4-step 10-fold serial dilutions of mixed standard *C. felis*  
231 cDNA. RT-qPCR was performed to measure the expression of vitellogenin in samples from  
232 male (n = 3) and female (n = 3) fed adult *C. felis*, and normalized using the best single  
233 reference genes (GAPDH, Ef), best two reference genes (GAPDH + Ef), best three reference  
234 genes (GAPDH + Ef + RPL19) or least stable reference gene (18S) as listed in the overall  
235 ranking of reference genes for this comparison (Table 2). First the R0 for each sample was  
236 calculated for each gene for each sample using the equation  $R0 = 1/(1+E)^{Cq}$ , then the  
237 normalized values were calculated by dividing Vit R0 by the reference gene R0 or geometric  
238 mean of R0 for normalization with multiple reference genes.

## 239 RESULTS

#### 240 *Impact of sample storage method on RNA quality*

241 The electropherograms for pierced larvae and adult samples are similar after 0, 3 and 10 days  
242 storage in RNAlater at room temperature (Fig. 1), with no appreciable accumulation of small  
243 RNA fragments visible. In contrast, degradation was clear in unpierced samples within 3  
244 days, particularly in larvae samples (Fig. 1B). By day 10 at room temperature the majority of  
245 large RNA transcripts appeared to be fragmented, demonstrating RNA quality had dropped  
246 significantly.

247 *PCR efficiencies and expression levels of candidate reference genes*

248 Primer efficiencies ranged from 83.5-97.5 %, with most primer sets having efficiency greater  
249 than 90 %. The DLAct primers had a lower efficiency than preferable (83.5 %) and would  
250 have been discarded based on normal acceptable efficiency criteria. However, the DLAct  
251 primers were still used in reference gene testing for comparison due to their prior use in a  
252 publication (Dreher-Lesnack et al. 2010). NADH primers were not used for further analysis  
253 due to their highly variable efficiency ( $E = 90.7 \%$ ,  $SD = 16.7 \%$ ).

254 Cq values across all treatment samples (Mean  $\pm$  SD,  $n = 30$ ) for the 9 analysed reference  
255 genes ranged from  $15.34 \pm 1.65$  (28S) to  $22.44 \pm 1.34$  ( $\alpha$ -Tubulin) (Fig. 2). GAPDH was the  
256 least variable reference gene tested across all samples (coefficient of variation (CV) = 3.45  
257 %), while 28S was the most variable (CV = 10.93 %). Several genes (18S, 28S, DLAct) had  
258 clear outlying values, which suggested instability (Fig. 2).

259 *Expression stability of reference genes across developmental stages*

260 Three software programs were used to rank the nine candidate reference genes in *C. felis* for  
261 their stability across different developmental stages (larvae  $n = 3$ , pupae  $n = 3$ , unfed adults  $n$   
262  $= 2$ , fed adults  $n = 3$ ; throughout the study  $n =$  number of pooled samples tested, each pool  
263 contained between 3 and 10 fleas) (Table 2). GeNorm ranked the genes based on their  
264 average expression stability (M), calculating this value with all genes included then removing  
265 the least stable gene and recalculating M until only two genes remain which cannot be further  
266 differentiated (Fig. 3). Ef and RPL19 were identified as the most stable genes by geNorm (M  
267  $= 0.132$ ) and 28S the least stable (M = 1.203) (Table 2, Fig. 3). However, all genes tested had  
268  $M < 1.5$  therefore can be considered stable enough to use as reference genes according to this  
269 analysis. A pairwise variation analysis between normalization factors  $V_n/V_{n+1}$  was also  
270 performed by geNorm to assess the minimal number of reference genes needed for accurate  
271 normalization. Pairwise variation ( $V$ )  $< 0.15$  indicates additional reference genes are  
272 unnecessary. For comparisons across all developmental stages  $V_{2/3} V = 0.048$ , indicating  
273 two reference genes are sufficient for normalization in this case (Fig. 4) and no significant  
274 benefit is gained by using  $> 2$  reference genes.

275 The best gene determined by Normfinder analysis for comparisons between developmental *C.*  
276 *felis* groups was RPL19 (SV = 0.270) and the best combination of two genes was actin and  
277 GAPDH (SV = 0.210) (Table 2). HSP and 28S were found to be the least stable genes, with

278 SV > 1 suggesting they were unsuitable for use as reference genes in *C. felis* studies (Table  
279 2).

280 Cq and efficiency values were input into Bestkeeper to produce descriptive statistics. The  
281 standard deviation  $\pm$  Crossing Point (SD  $\pm$  CP) can be used to rank stability. Under this  
282 criteria 18S was ranked as the most stable *C. felis* gene (SD  $\pm$  CP = 0.54), followed by  
283 GAPDH (SD = 0.63) and Ef (SD = 0.76). HSP was the least stable gene (SD = 1.89) and  
284 considered too unstable for use as a reference gene as it had SD > 1.

285 The rankings for each program were combined using a points-based system to estimate an  
286 overall ranking of reference gene stability. This ranking found Ef, RPL19 and Act to be the  
287 most stable genes across *C. felis* developmental stages and 28S and HSP to be the least stable  
288 candidates (Table 2).

#### 289 *Expression stability of reference genes across sexes*

290 Comparing the stability of candidate reference genes between male (n = 3) and female (n = 3)  
291 fed *C. felis* adults, geNorm ranked GAPDH and RPL19 as the most stable (M = 0.112) (Table  
292 2, Fig. 3). 18S was the least stable gene based on this comparison; although all genes had M  
293 < 1.5 therefore can be considered as potentially suitable reference genes in *C. felis*. Pairwise  
294 comparison of normalization factors suggested two genes are sufficient for accurate  
295 normalization (V = 0.049) (Fig. 4). Normfinder ranked GAPDH as the most stable gene (SV  
296 = 0.144), Act and Ef as the best combination of two genes (SV = 0.111) and DLAct the least  
297 stable (SV = 0.510) (Table 2). DLAct was ranked as the most stable gene by Bestkeeper (SD  
298 = 0.51), while suggesting 18S, HSP and  $\alpha$ Tub are unsuitable as reference genes (SD > 1). The  
299 combined overall ranking placed GAPDH, Ef and RPL19 as the most stable candidate  
300 reference genes across *C. felis* and 18S as the least stable (Table 2).

#### 301 *Expression stability of reference genes across feeding statuses*

302 GeNorm ranked Act and Ef as the most stable genes across feeding statuses (unfed adults n =  
303 2, fed adults n = 4) (M = 0.112) (Table 2). Two genes were found to be sufficient for  
304 normalization (Fig. 4). 18S was the least stable gene according to both geNorm and  
305 Normfinder. Normfinder placed GAPDH as the most stable gene (SV = 0.092) and GAPDH  
306 and RPL19 to be the best combination of two genes (SV = 0.065). Bestkeeper estimated 28S  
307 and DLAct as the most and least stable genes respectively. Each candidate met the  
308 requirements to be classed as a suitable reference gene by all programs in this comparison.

309 The overall points-system ranking placed RPL19, GAPDH and Ef as the most stable  
310 reference genes across fed and unfed *C. felis* adults and 18S as the least stable candidate.

### 311 *Expression stability of reference genes across insecticide treatment statuses*

312 Stability of reference genes across treated (1  $\mu$ M selamectin, n = 3) and untreated (n = 3) fed  
313 adult *C. felis* was investigated. Act and RPL19 were the most stable candidates according to  
314 geNorm (M = 0.104) (Table 2, Fig. 3). Bestkeeper also ranked these as the top two reference  
315 genes (Table 2). Two candidates were estimated to be sufficient for accurate normalization  
316 (V = 0.150) (Fig. 4). Ef (SV = 0.035) or a combination of Ef and  $\alpha$ Tub (SV = 0.042) were the  
317 best candidates according to Normfinder (Table 2). All programmes ranked 18S as the least  
318 stable gene across treatment statuses, with geNorm and Bestkeeper both classing it as  
319 unsuitable for use as a reference gene. Bestkeeper also found  $\alpha$ Tub, DLAct and 28S to be  
320 unsuitable candidates, perhaps because samples within this group account for several of the  
321 outliers seen in Fig. 2, which are likely to lead to a high standard deviation. The most stable  
322 genes in the overall ranking were Act, RPL19 and GAPDH, with 18S as the least stable  
323 candidate by this estimate.

### 324 *Validation of reference genes – a case study in vitellogenin C levels across sexes*

325 In all cases vitellogenin C was found to be upregulated in females relative to males, with  
326 reported fold-changes ranging from 8.46x to 12.32x (Fig. 5). Normalization with the two best  
327 reference genes individually led to disagreement in fold-change (GAPDH = 8.46x, Ef =  
328 11.08x), whereas results were more consistent when using 2 or 3 reference genes in  
329 combination ( $9.69x \pm 1.07$  &  $9.32x \pm 0.80$ ) respectively). The coefficient of variation of the  
330 normalised fold change was much higher when using the least stable gene (18S) to normalise  
331 (37.98 %) compared to any of the combinations of single or multiple more stable genes,  
332 where the coefficient of variation ranged from 8.60-12.70 %.

## 333 DISCUSSION

334 RNA samples are highly susceptible to breakdown from endogenous RNases following  
335 collection. RNAlater, a high density salt solution, acts to stabilise RNA by preventing action  
336 of such RNases. In order to work effectively RNAlater must enter tissues (Chen et al. 2007)  
337 but external structures, such as fine hairs on the surface of many arthropods, can prevent the  
338 solution from contacting internal tissues. Thus, it is often necessary to penetrate the sample  
339 tissues for proper exposure to RNAlater. Piercing individual cat fleas is a relatively laborious

340 process due to their small size and could dissuade some potential collaborators (e.g.  
341 veterinarians, kennel staff, the general public) from collecting fleas for downstream gene  
342 expression work. Thus, it was investigated if piercing is actually necessary for maintenance  
343 of RNA integrity by RNAlater. This study found that penetrating *C. felis* specimens is  
344 essential for preservation of RNA, with degradation clearly apparent in unpierced larvae and  
345 adult samples after even 3 days at room temperature (Fig. 1). However when specimens were  
346 pierced prior to submergence in RNAlater they could be stored at room temperature for up to  
347 10 days with little degradation visible on electropherogram traces. A small peak at around 25  
348 seconds was visible in pierced adult electropherograms after 3 and 10 days, representing  
349 small RNAs which could be indicative of a small amount of degradation. Thus, samples  
350 could be shipped at ambient temperature nationally and internationally for collaboration  
351 between research groups, if pierced upon collection and placed in RNAlater. Such an  
352 approach may be particularly useful when fleas are to be collected by veterinary practices or  
353 pet owners before being passed onto the research organisation. However if a particularly  
354 sensitive technique is to be utilized such as RNASeq it may still be beneficial to freeze  
355 samples before transportation on dry ice.

356

357 Reference genes which are stable across experimental conditions are essential to reliable  
358 interpretation of RT-qPCR data. Although several studies have used RT-qPCR to look at *R.*  
359 *felis* bacterial replication within the cat flea (Henry et al. 2007; Obhiambo et al. 2014), few  
360 have utilised the technique to study endogenous cat flea gene expression (Dreher-Lesnick et  
361 al. 2010). Past historical “housekeeping genes” have often been used in arthropod studies  
362 without proper validation. Recently, systematic screening of candidate reference genes has  
363 been performed for many insect species (Scharlaken et al. 2008; Teng et al. 2012; Li et al.  
364 2013; Omondi et al. 2015; Shakeel et al. 2015), with many of these studies highlighting the  
365 importance of validating references in all experimental conditions and tissues of interest. In  
366 this study we systematically assessed ten candidate reference genes for stability within 4  
367 groups of *C. felis*: developmental stages, sexes, feeding statuses and insecticide-treatment  
368 statuses. Transcripts commonly used in other insect species were selected for comparison  
369 (Scharlaken et al. 2008; Li et al. 2013; Zhai et al. 2014; Tan et al. 2015).

370

371 Three programs were used to estimate the stability of the candidate reference genes, geNorm,  
372 Normfinder and Bestkeeper. Each program uses a different algorithm to assess stability,

373 leading to differences in the rankings between programmes. This was particularly apparent  
374 for Bestkeeper in this study, which often highlighted as the best gene a candidate which was  
375 ranked low by other programmes (Table 2). To give an easy guide to stable reference genes  
376 an overall ranking was produced for each comparison. This overall ranking showed GAPDH,  
377 RPL19 and Ef to rank highly in all comparisons (Table 2). Ribosomal proteins, GAPDH and  
378 Ef have all been characterised recently as stable reference genes in other arthropod species  
379 (Scharlaken et al. 2008; Teng et al. 2012; Li et al. 2013; Omondi et al. 2015; Shakeel et al.  
380 2015). While it is important to assess stability of references in specific experimental  
381 conditions, GAPDH, RPL19 and Ef would be recommended as reference genes for any of the  
382 comparisons tested here in *C. felis*.

383

384 The use of unstable reference genes can have a large impact on the interpretation of RT-  
385 qPCR results (Kidd et al. 2007; Boda et al. 2008; Kosir et al. 2010). To validate the ranking  
386 of our candidate genes levels of vitellogenin C in male and female fed adult *C. felis* were  
387 investigated, using the best three (GAPDH + Ef + RPL19), two (GAPDH + Ef) or single  
388 (GAPDH, Ef) genes and the least stable (18S). Vitellogenin C levels were found to be  
389 approximately 9-fold higher in females compared to males. While all normalization strategies  
390 demonstrated an increase in vitellogenin C in females, the estimated fold change varied from  
391 8.5-fold to 12.3-fold (Fig. 5). Using the least stable gene for normalization gave a high  
392 coefficient of variation (37.98 %) compared to the other normalization strategies (CV 8.60-  
393 12.70 %), demonstrating the uncertainty introduced by use of an inappropriate reference  
394 gene. This is particularly important when trying to detect small changes in gene expression  
395 between samples, where instability of a reference gene could lead to misinterpretation of  
396 results (Omondi et al. 2015). Use of two or three reference genes generated a more consistent  
397 fold change estimate (9.69-fold & 9.32-fold respectively), with single reference genes  
398 generating different estimates (GAPDH = 8.46x, Ef = 11.08x). This highlights the importance  
399 of using multiple reference genes for accurate normalization.

400

401 The present study provides insight into sample preparation and reference genes suitable for  
402 use across a variety of conditions for *C. felis* specimens. In summary, our findings  
403 recommend piercing of *C. felis* before placing in an RNA-stabilizing solution and storing at  
404 room temperature and that two reference genes selected from GAPDH, Ef and RPL19 are  
405 suitable and suffice for accurate gene expression studies in *C. felis* in the given experimental

406 conditions. This paves the way for new investigations into *C. felis* gene expression, opening  
407 new avenues for the research community to utilise to find ways to tackle this common pest.

408

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411

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415

#### 416 CONFLICT OF INTEREST

417 None

418

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532 **Table 1. Candidate reference genes assessed for stability.**533 <sup>a</sup> T<sub>m</sub>, melting temperature for oligos; <sup>b</sup> E, efficiency of primers, assessed by standard curve

534 slope. SD calculated for efficiencies between runs.

Transcript Name	Function	Oligo Sequence	Product size (bp)	T <sub>m</sub> (°C) <sup>a</sup>	E ±SD (%) <sup>b</sup>
18S ribosomal RNA gene (18S)	Structural protein	F: CCTGCGGCTTAATTTGACTC	135	59.8	94.1±5.1
	in ribosome	R: AGACAAATCGCTCCACCAAC		60.1	
28S ribosomal RNA gene (28S)	Structural protein	F: AAACGGTCCTTGTGACTTGG	136	60	92.7±2.6
	in ribosome	R: TCTGAGCTGACCGTTGAATG		60	
β-Actin (Act)	Cytoskeletal	F: AGGAATTGCTGACCGTATGC	139	60.1	97.5±1.8
	structure	R: TTGGAAGGTGGATAGGGATG		59.7	
Muscle specific actin (DLAct)	Cytoskeletal	F: GGTCGGTATGGACAAAAGGAC	367	59.9	83.5±1.3
	structure	R: GTAGATTGGGACGGTGTGAGAGAC		62.3	
Elongation factor 1 α (Ef)	Translation	F: TCGTACTGGCAAATCCACAG	145	59.7	95.3±4.8
		R: CATGTCACGGACAACGAAAC		60	
Glyceraldehyde 3 phosphate dehydrogenase (GAPDH)	Glycolysis	F: ACCCAAAAGACTGTGGATGG	117	59.8	91.4±1.5
		R: CGGAATGACTTTGCCTACAG		58.4	
Heat shock protein 22 (HSP)	Stress response	F: ACCCAATGCGTCTTATGGAC	103	59.8	93.7±2.3
		R: TAATAACCGCCACGGAAGAG		60.1	
NADH dehydrogenase/ubiquinone reductase (NADH)	Respiratory chain enzyme	F: GTCGCTGGTGTAGATGATCTTG	133	59.8	90.7±16.7
		R: TTCGACGTTAAGCACCACAG		59.9	
60S ribosomal protein L19 (RPL19)	Structural protein	F: TACAGCTAATGCCCGTACACC	72	60	91.7±1.1
	in ribosome	R: TTCAACAAACGCCTCAGGAC		61.2	
α-Tubulin (αTub)	Cytoskeletal	F: AACTATTGGAGGCGGTGATG	125	60	91.6±3.4
	structure	R: TTGACGGTATGTTCCAGTGC		59.6	
Vitellogenin	Reproduction	F: CAAGAATCCAGCTCCTCCAG	204	59.9	91.2±2.0
		R: ACGGATGCTGAAGCAGAGTT		60	

535

536 **Table 2. Rankings of candidate reference genes**

537 M = average expression stability (geNorm), SV = stability value (Normfinder), SD ±  
 538 CP = standard deviation ± crossing point (Bestkeeper), \* = not considered a suitable  
 539 reference gene by this programme. Overall ranking is based on a points-based  
 540 system to combine the rankings from all programmes used. All rankings are stated  
 541 from most stable (1) to least stable (10).

542

**Developmental Stages**

Ranking	GeNorm	M	Normfinder	SV	Bestkeeper	SD ± CP	Overall Ranking
1	Ef/ RPL19	0.132	RPL19	0.27	18S	0.54	Ef
2	-	-	Ef	0.276	GAPDH	0.63	RPL19
3	Act	0.149	Act	0.29	Ef	0.76	Act
4	aTub	0.257	GAPDH	0.318	Act	0.8	GAPDH
5	GAPDH	0.441	aTub	0.365	RPL19	0.88	18S
6	DLAct	0.626	DLAct	0.558	DLAct	0.91	aTub
7	18S	0.845	18S	0.902	28S	0.97	DLAct
8	HSP	1.034	HSP	1.054*	aTub	0.98	28S / HSP
9	28S	1.203	28S	1.145*	HSP	1.89*	-

**Males vs Females**

Ranking	GeNorm	M	Normfinder	SV	Bestkeeper	SD ± CP	Overall Ranking
1	GAPDH/ RPL19	0.114	GAPDH	0.144	DLAct	0.51	GAPDH
2	-	-	Ef	0.188	Ef	0.54	Ef
3	Ef	0.142	Act	0.197	RPL19	0.61	RPL19
4	Act	0.196	RPL19	0.216	GAPDH	0.7	Act
5	HSP	0.408	HSP	0.412	28S	0.76	28S / DLAct / HSP
6	aTub	0.497	28S	0.414	Act	0.78	-
7	28S	0.629	aTub	0.469	18S	1.21*	-
8	DLAct	0.73	18S	0.491	HSP	1.23*	aTub
9	18S	0.83	DLAct	0.51	aTub	1.34*	18S

**Fed Adults vs Unfed Adults**

Ranking	GeNorm	M	Normfinder	SV	Bestkeeper	SD ± CP	Overall Ranking
1	Act / Ef	0.112	GAPDH	0.092	28S	0.27	RPL19
2	-	-	RPL19	0.147	GAPDH	0.29	Ef / GAPDH
3	RPL19	0.141	aTub	0.16	RPL19	0.42	-
4	HSP	0.163	Ef	0.206	Ef	0.47	aTub
5	aTub	0.195	DLAct	0.23	HSP	0.51	Act
6	GAPDH	0.221	Act	0.236	aTub	0.52	HSP
7	DLAct	0.266	HSP	0.241	Act	0.54	28S
8	28S	0.383	28S	0.431	18S	0.57	DLAct
9	18S	0.489	18S	0.491	DLAct	0.71	18S

**Insecticide Treated vs Untreated**

Ranking	GeNorm	M	Normfinder	SV	Bestkeeper	SD ± CP	Overall Ranking
1	Act / RPL19	0.104	Ef	0.035	Act	0.22	Act / RPL19
2	-	-	aTub	0.075	RPL19	0.25	-
3	GAPDH	0.337	HSP	0.135	GAPDH	0.45	Ef
4	HSP	0.459	DLAct	0.138	Ef	0.63	GAPDH
5	Ef	0.502	GAPDH	0.252	HSP	0.66	HSP
6	aTub	0.597	RPL19	0.408	aTub	1.07*	aTub
7	DLAct	0.734	Act	0.416	DLAct	1.45*	DLAct
8	28S	1.115	28S	0.581	28S	2.21*	28S
9	18S	1.533*	18S	0.952	18S	2.82*	18S

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549 **Fig. 1 Electropherogram assessment of RNA quality for pierced and unpierced *C. felis***  
550 **specimens stored at room temperature for 0, 3 or 10 days.**

551 Total RNA was extracted from pierced or unpierced *C. felis* larvae (Fig. 1A) or adults (Fig.  
552 1B) which had been stored in RNAlater at room temperature for 0, 3 or 10 days. 40-120 ng of  
553 RNA were run on the Agilent Bioanalyzer 2100 microfluidics gel analysis platform to  
554 determine RNA quality.

555

556 **Figure 2. Average cycle thresholds of candidate reference genes.**

557 The boxplot represents median, and indicates 25th and 75th percentile. Whiskers represent  
558 the 10th and 90th percentiles. Black dots indicate outliers. Cq values for all tested samples (n  
559 = 30) across all groups (*C. felis* developmental groups, sexes, feeding statuses and insecticide  
560 treatment statuses).

561

562 **Figure 3. Average expression stability of candidate reference genes**

563 Values for average expression stability (M) as calculated by geNorm (v. 3.4) by pairwise  
564 comparison and stepwise exclusion of the least stable reference gene, for four group  
565 comparisons: (A) *C. felis* developmental stages (larvae, pupae, unfed adults, fed adults); (B)  
566 feeding statuses (unfed and fed adults); (C) sexes (males and females); (D) Insecticide-  
567 treatment statuses (treated with 1  $\mu$ M selamectin or untreated fed adults).

568

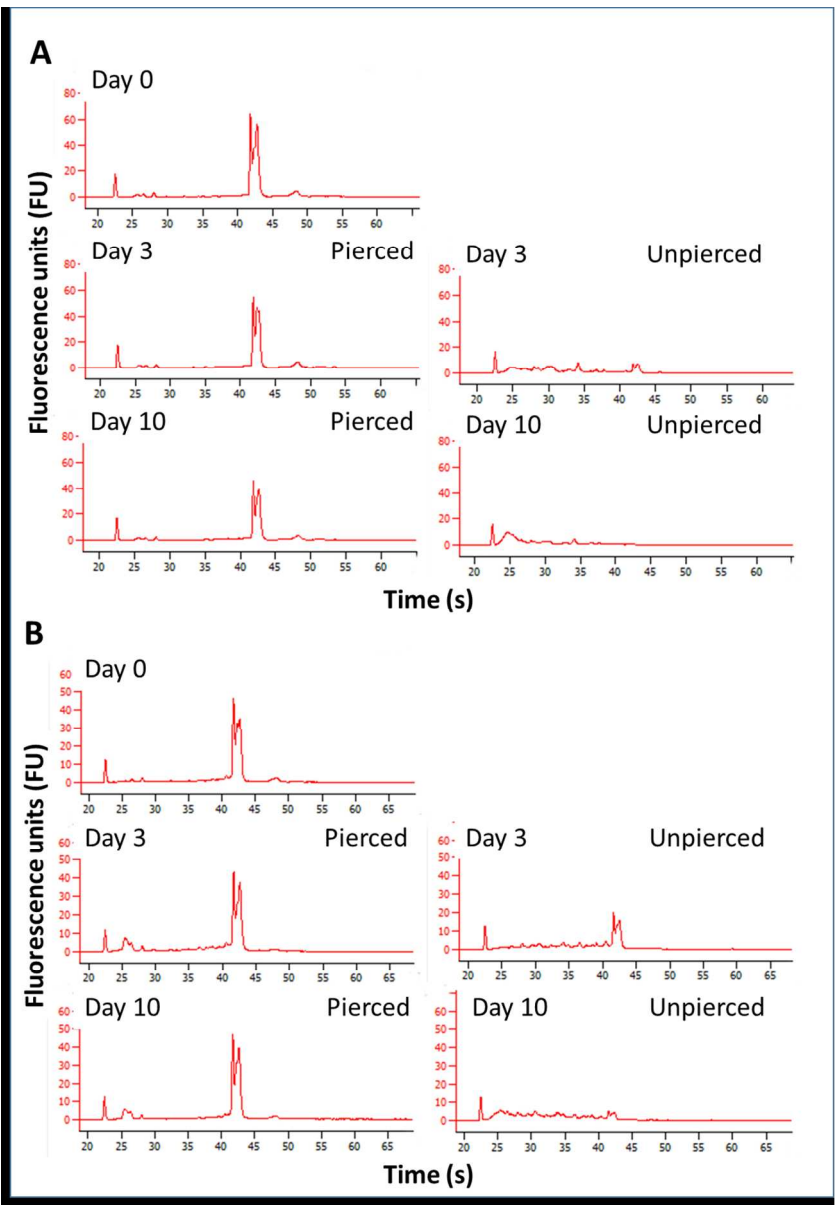
569 **Figure 4. Pairwise variation values for assessment of necessary number of**  
570 **reference genes**

571 geNorm (v. 3.4) calculated pairwise variation (V) for assessment of sufficient number of  
572 reference genes for accurate normalization in each of four group comparisons of *C. felis*:  
573 developmental stages (larvae, pupae, unfed adults, fed adults); feeding statuses (unfed and  
574 fed adults); sexes (males and females); insecticide-treatment statuses (treated with 1  $\mu$ M  
575 selamectin or untreated fed adults).  $V < 0.15$  indicates inclusion of a further reference gene is  
576 of negligible benefit.

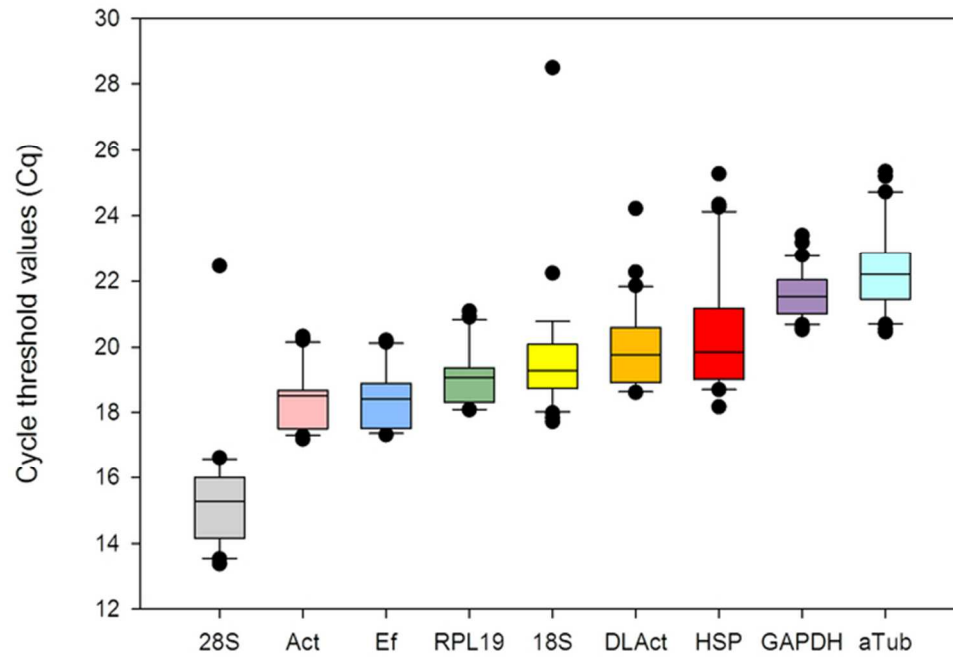
577

578 **Figure 5. Validation of reference genes by testing vitellogenin C expression levels.**

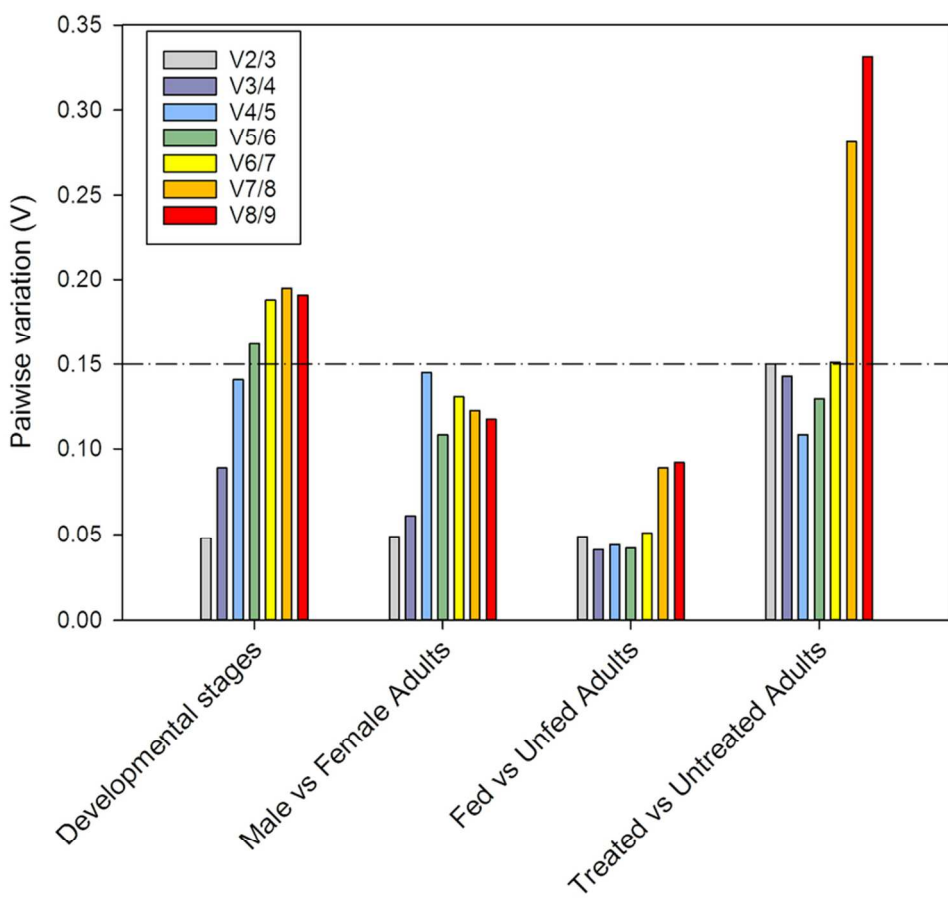
579 Vitellogenin C levels were measured in female relative to male fed *C. felis* adults.  
580 Vitellogenin C expression levels were assessed relative to single most (GAPDH, Ef) or least  
581 (18S) stable reference genes or combinations of the two (Ef + GAPDH) or three (Ef +  
582 GAPDH + RPL19) most stable reference genes. Data are means  $\pm$  SEM, n = 3.



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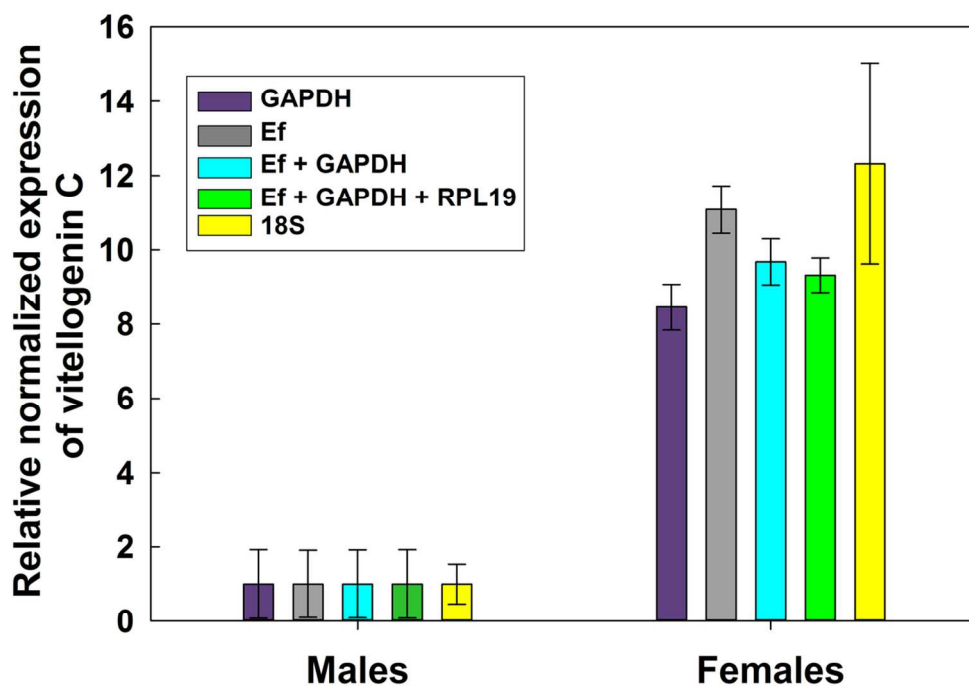


59x44mm (300 x 300 DPI)



80x80mm (300 x 300 DPI)





57x41mm (600 x 600 DPI)