Selection of suitable reference genes for gene expression studies in myxosporean (Myxozoa, Cnidaria) parasites

Anush Kosakyan1*, Gema Alama-Bermejo1,2,3, Pavla Bartošová-Sojková1, Ana Born-Torrijos1, Radek Šíma1, Anna Nenarokova1,4, Edit Eszterbauer5, Jerri Bartholomew2 & Astrid S. Holzer1

Myxozoans (Cnidaria: Myxozoa) are an extremely diversified group of endoparasites some of which are causative agents of serious diseases in fish. New methods involving gene expression studies have emerged over the last years to better understand and control myxozoan diseases. Quantitative RT-PCR is the most extensively used approach for gene expression studies. However, the accuracy of the results depends on the normalization of the data to reference genes. We studied the expression of eight commonly used reference genes, adenosylhomocysteinase (AHC1), beta actin (ACTB), eukaryotic translation elongation factor 2 (EF2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1), DNA-directed RNA polymerase II (RPB2), 18S ribosomal RNA (18S), 28S ribosomal RNA (28S) across different developmental stages of three myxozoan species, Sphaerospora molnari, Myxobolus cerebralis and Ceratonova shasta, representing the three major myxozoan lineages from the largest class Myxosporea. The stable reference genes were identified using four algorithms: geNorm, NormFinder, Bestkeeper and ΔCq method. Additionally, we analyzed transcriptomic data from S. molnari proliferative and spore-forming stages to compare the relative amount of expressed transcripts with the most stable reference genes suggested by RT-qPCR. Our results revealed that GAPDH and EF2 are the most uniformly expressed genes across the different developmental stages of the studied myxozoan species.

Myxozoans are a cnidian group of obligate parasites documented mainly from fish in marine and freshwater habitats. These microscopic endoparasites have a two-host life cycle that involves an invertebrate (annelids and bryozoans) and a vertebrate host (mostly fish, few are known from other vertebrates) where infectious actinospores and myxospores are formed, respectively, serving as transmission stages in aquatic habitats1,2. The current classification of the Myxozoa into classes mainly reflects spore morphology and invertebrate host types. Taxa are ranked in the class Myxosporea Bütschli 1881 according to their hardened shell valves and annelid definitive hosts while the ones with soft spore valves and bryozoan definitive hosts are representatives of the second class Malacosporea Canning, Curry, Feist, Longshaw et Okamura 2000. Myxosporea represents the largest class comprising 19 families and 67 genera while Malacosporea have only one family and two genera3.

Myxosporeans have received considerable attention since some of them are reported to cause severe fish diseases. These parasites can have a strong impact on wild and cultured fish worldwide by reducing fillet marketability and causing important mortalities in fish populations4-8. Given the fact that aquaculture is one of the fastest growing food sectors9,10 comprising an ample part of global food production, economic losses caused by parasites such as myxosporeans are of major concern11,12. Furthermore, disease severity has been linked to increasing water
temperatures (i.e. in Ceratonova shasta\textsuperscript{13,14}), predicting emerging numbers of these organisms in the future as a result of climate change.

In this study we have focused on three myxosporean species, Sphaerospora molnari, Myxobolus cerebralis and Ceratonova shasta that belong to three (sphaerosporids, oligochaete-infecting and polychaete-infecting lineages\textsuperscript{15}) out of four main myxozoan phylogenetic lineages and transcriptomic data are available (16, Alama-Bermejo \textit{et al.} submitted, Hartigan \textit{et al.} submitted). These species are considered serious pathogens for highly commercialized fishes such as cyprinids and salmonids which represent a significant proportion of worldwide aquaculture production. \textit{Sphaerospora molnari} causes respiratory and osmoregulatory failure in host's gill epithelia\textsuperscript{17}, while proliferative blood stages induce a massive systemic inflammatory response\textsuperscript{18}. It was also shown that \textit{S. molnari} may be an important co-factor for swim bladder inflammation in carp, the disease responsible for up to 100% mortalities of carp fingerling stocks in Central Europe\textsuperscript{19}. The invertebrate definitive host of \textit{S. molnari} is unknown. \textit{Myxobolus cerebralis} causes serious damage to farmed and wild salmonid fish populations worldwide. It is responsible for whirling disease, a condition caused by infection of the hosts central nervous system and cartilage resulting high mortalities\textsuperscript{8}. The life cycle of \textit{M. cerebralis} involves the oligochaete \textit{Tubifex tubifex} and salmonids as vertebrate hosts. \textit{Ceratonova shasta} is a serious pathogen of wild and cultured salmonids in the Pacific Northwest of North America, including endangered Coho and Chinook salmons. \textit{C. shasta} causes intestinal enteritis with up to 100% mortality in certain populations. The definitive invertebrate host of \textit{C. shasta} is the freshwater polychaete \textit{Manayunkia speciosa}\textsuperscript{20}.

There are currently no disease control methods for myxozoans in general, as no vaccines or commercial treatments for fish destined for human consumption are available. In order to design efficient methods for prediction and control of myxozoan diseases it is important to explore genes that these parasites use for invasion of and survival within their host, e.g. genes involved in immune evasion, since they are potential candidates for targeted antiparasitic treatments and vaccine development. As a result of this need, the number of gene expression studies are presently expanding considerably in order to predict and control such functional genes.

RT-qPCR (reverse transcriptase quantitative PCR) is one of the most rapidly incorporated techniques in scientific studies. Its application in mRNA quantification has grown from ~8% to ~73–88% in the last decade\textsuperscript{21}.

Being considered highly sensitive, RT-qPCR is one of the most extensively used approaches for gene expression studies in all organisms\textsuperscript{22–24}. To achieve accurate gene expression results, it is critical that RT-qPCR results are normalized to an internal control, since gene expression can be influenced by different factors, i.e. variation in the amount of starting material, differences in RNA contents between cells or developmental stages, technical variability, and transcription efficiency. Traditionally, housekeeping genes (hereafter HKGs) are used as internal control. HKGs are present in all cell types because they are necessary for basic cell survival. HKGs commonly used as internal controls include beta actin, glyceraldehyde-3-phosphate dehydrogenase, several ribosomal genes such as 18S rRNA, 28S rRNA and eukaryotic elongation factors. Due to their key roles in metabolism, cytoskeleton and ribosome structure, the mRNA/rRNA synthesis of these genes was considered to be stable or uniformly expressed in various tissues, during ontology and development, even under different treatments\textsuperscript{19–21} and thus these genes were considered good reference genes (hereafter RGs).

However, it was shown that HKGs independent of organism do not always perform as good RG, and their expression may be differentially regulated and vary under certain experimental conditions. That is why it is highly recommended to validate the HKGs for each organism and study before performing gene expression studies\textsuperscript{22}.

For cnidarians in general, data on RGs are scarce, although few differential expression studies were performed\textsuperscript{26–30}. For myxozoans, comprehensive gene expression studies are inexistent and only three reports study parasite gene expression\textsuperscript{31–33} and mainly rely on RGs that were “used in previous publications” (e.g. \textsuperscript{31}), or the validation of RGs was focused on a limited part of parasite development (e.g. early intrapiscine development studied by Eszterbauer \textit{et al.}\textsuperscript{32}). Myxozoans are some of the oldest metazoan parasites with an extremely accelerated evolutionary rate and high heterogeneity across genes\textsuperscript{15}, and likely functional derivation of genes. Therefore, it can be expected that genes that serve as RGs in other organisms are not constantly expressed during the complex life cycle of myxozoans and across the different developmental stages, within the vertebrate and invertebrate hosts. 18S rDNA is presently the most commonly used gene region for phylogenetic studies\textsuperscript{15} and especially for \textit{Ceratonova shasta}\textsuperscript{31,32}, predicting emerging numbers of these organisms in the future as a result of climate change.

Material and Methods

Parasite collection. For each species different developmental stages (\textit{S. molnari}) and different life cycle stages (\textit{M. cerebralis} and \textit{C. shasta}) were isolated from fish and definitive worm hosts (Table 1).

\textit{S. molnari} proliferative, presporogenic blood stages were collected from a laboratory line that has been cycled (2 + years) from fish to fish by intraperitoneal injection of parasites into specific parasite-free (SPF) common carp (\textit{Cyprinus carpio}) (methodology detailed in\textsuperscript{18}). \textit{S. molnari} blood stages (\textit{n} = 5 fish) were concentrated and co-isolated with host white blood cells from whole blood of carp, by centrifugation for 5 minutes at 3500 rpm in heparinized hematocrit tubes\textsuperscript{18}. Spore-forming stages (infected gills, \textit{n} = 5) were obtained from carp held at the recirculation system of the Research Institute for Fisheries and Aquaculture (Szarvas, Hungary).

\textit{M. cerebralis} actinospores used for exposure trials originated from \textit{Tubifex tubifex} cultures maintained in the laboratory of the Institute for Veterinary Medical Research (IVMR), Budapest, Hungary, over several years. SPF rainbow trout, \textit{Onchorhynchus mykiss} (Kamloops strain) was obtained from the Lillafüred Trout Hatchery, Hungary (yolk sac stage) and reared at the IVMR. Fish were infected individually with 5000 freshly filtered
actinospores according to37. From infected fish, pieces of skulls containing myxospores and sporogonic plasmodia (spore-forming stages) were collected 90 days’ post exposure (n = 4). Laboratory T. tubifex cultures were exposed with spores isolated from the head cartilage as per38. Worms (n = 4) infected with triactinomyxon spore-forming stages were collected 100 days’ post exposure.

The species composition of naïve worm cultures was regularly checked by DNA sequencing and microscopy, and worm specimens with long hair chaetae (which all belong to Tubifex tubifex s.l. in the culture) were selected for individual exposure.

Ceratonova shasta was collected from ascitic fluid of the abdominal cavity and from infected intestines of rainbow trout infected with genotype IIR (n = 3). Naïve rainbow trout were from Roaring River Hatchery strain (Scio, OR, Oregon Department of Fish and Wildlife) and they were infected by an intraperitoneal injection of ascites collected from an infected rainbow trout that was previously exposed in the Williamson River, Oregon, USA. Fish were held at 18 °C in 100 L tanks at the Aquatic Animal Health Laboratory at Oregon State University (AAHL, OR, Oregon Department of Fish and Wildlife) and they were infected by an intraperitoneal injection40.

Manayunkia sp. worms, was isolated using guanidine/thiocyanate/phenol/chloroform extraction method43 to ensure higher concentrations of RNA compared to the column-based RNA extraction methods. A DNase digestion step ensuring elimination of genomic DNA was included into the protocol of the Nucleospin RNA Kit (manufacturer’s instructions). RNA from Manayunkia worms was isolated using guanidine/thiocyanate/phenol/chloroform extraction method43 to ensure higher concentrations of RNA compared to the column-based RNA extraction methods. A DNase digestion step ensuring elimination of genomic DNA was included into the protocol of the Nucleospin RNA Kit (manufacturer’s instructions). For Manayunkia samples, DNA was removed using the DNAFree Kit (Invitrogen). RNA concentration and purity were checked using a Nano Drop - 1000 Spectrophotometer (Thermo Fisher Scientific Inc.). All RNA samples with 260/280 ratio in range of 1.9–2.0, and 260/230 ratio in range of 2.0–2.4 were chosen for cDNA synthesis. Approximately 500 ng RNA was used as an input for synthesis of 20 µl of cDNA using the Transcriptor High Fidelity cDNA synthesis Kit (Roche) following the manufacturer’s protocol.

Table 1. Sampling details of selected parasites across different developmental stages and non-infected host used as control.

| Parasite species | Host species | Origine of samples | Parasite developmental stage | Host tissue | Number of samples |
|------------------|--------------|--------------------|-----------------------------|-------------|------------------|
| Sphaerospora molnari | Cyprinus carpio | Malá Outrata pond, CZ | presporogonic blood stage | blood | 5 |
| Sphaerospora molnari | Cyprinus carpio | Malá Outrata pond, CZ | non infected | blood | 2 |
| Sphaerospora molnari | Cyprinus carpio | Szarvas, HU | sporogonic stage | gills | 5 |
| Sphaerospora molnari | Cyprinus carpio | Szarvas, HU | non infected | gills | 2 |
| Myxobolus cerebralis | Oncorhynchus mykiss | Inst for Veterinary Med Res, Budapest, HU | sporogonic stage | cartilage (head) | 4 |
| Myxobolus cerebralis | Oncorhynchus mykiss | Inst for Veterinary Med Res, Budapest, HU | non infected | cartilage (head) | 2 |
| Myxobolus cerebralis | Tubifex tubifex | Inst for Veterinary Med Res, Budapest, HU | triactinomyxon stage | whole worm | 4 |
| Myxobolus cerebralis | Tubifex tubifex | Inst for Veterinary Med Res, Budapest, HU | non infected | whole worm | 2 |
| Ceratonova shasta | Oncorhynchus mykiss | Roaring River Hatchery, Scio, OR, USA | mix of presporogonic and sporogonic stages | intestine | 3 |
| Ceratonova shasta | Oncorhynchus mykiss | Roaring River Hatchery, Scio, OR, USA | non infected | intestine | 2 |
| Ceratonova shasta | Oncorhynchus mykiss | Roaring River Hatchery, Scio, OR, USA | mix of presporogonic and sporogonic stages | ascites | 3 |
| Ceratonova shasta | Oncorhynchus mykiss | Roaring River Hatchery, Scio, OR, USA | non infected | ascites | 2 |
| Ceratonova shasta | Manayunkia sp. | Fryer Aquatic Animal Health Lab (OSU), USA | tetractinomyxon stage | whole worm | 3 |
| Ceratonova shasta | Manayunkia sp. | Fryer Aquatic Animal Health Lab (OSU), USA | tetractinomyxon stage | whole worm | 2 |

Ethics statement. Fish manipulation and sampling techniques were performed in accordance with Czech legislation (Protection of Animals Against Cruelty Act No. 246/1992) and approved by the Czech Ministry of Agriculture. Rainbow trout sampling at Oregon State University (OSU) was carried out in accordance with the recommendations of OSU - Institutional Animal Care and Use Committee (IACUC). The protocol was approved by ACUP #4666. For rainbow trout exposure to M. cerebralis, the Hungarian Scientific Ethical Committee on Animal Experimentation provided approval (PEI/001/4087–4/2015).

RNA extraction and reverse transcription. Total host + parasite RNA for all samples with exception of Manayunkia worms, was isolated using the Nucleospin RNA Kit (Macherey-Nagel) following manufacturer’s instructions. RNA from Manayunkia worms was isolated using guanidine/thiocyanate/phenol/chloroform extraction method43 to ensure higher concentrations of RNA compared to the column-based RNA extraction methods. A DNase digestion step ensuring elimination of genomic DNA was included into the protocol of the Nucleospin RNA Kit (manufacturer’s instructions). For Manayunkia samples, DNA was removed using the DNAFree Kit (Invitrogen). RNA concentration and purity were checked using a Nano Drop - 1000 Spectrophotometer (Thermo Fisher Scientific Inc.). All RNA samples with 260/280 ratio in range of 1.9–2.0, and 260/230 ratio in range of 2.0–2.4 were chosen for cDNA synthesis. Approximately 500 ng RNA was used as an input for synthesis of 20 µl of cDNA using the Transcriptor High Fidelity cDNA synthesis Kit (Roche) following the manufacturer’s protocol.
Table 2. Details of selected candidate reference genes.

| Gene Ids | Protein encoded | Generally accepted function | Accepted reference gene for | Used as RG for myxozoans |
|----------|-----------------|-----------------------------|-----------------------------|--------------------------|
| ACTB     | Beta actin      | Cytoskeletal structural protein | Commonly accepted reference gene for cnidarians | Myxobolus cerebralis |
| AHC1     | Adenosylhomocysteinase | Homocysteine synthesis protein | Corals | |
| EF2      | Eukaryotic Translation Elongation Factor 2 | Nascent protein synthesis protein | Commonly accepted reference gene for metazoan | |
| GAPDH    | Glyceraldehyde 3-Phosphate Dehydrogenase | Metabolic protein (glycolytic enzyme) | Commonly accepted reference gene for metazoan | |
| HPRT1    | Hypoxanthine-Guanine Phosphoribosyltransferase 1 | Purine nucleotide synthesis protein | Commonly used for humans | |
| RPB2     | DNA-directed RNA polymerase II | RNA polymerase II transcription machinery protein | Humans | |
| 18S rRNA | 18S ribosomal RNA gene SSU | Ribosomal structural protein | Commonly used reference gene for cnidarians | Myxobolus cerebralis |
| 28S rRNA | 28S ribosomal RNA gene LSU | Ribosomal structural protein | Commonly used reference gene for cnidarians | |

Candidate reference gene selection and data mining. A list of eight commonly used cnidarian and other metazoan candidate reference genes were selected for this study: adenosylhomocysteinase (AHC1), beta actin (ACTB), eukaryotic translation elongation factor 2 (EF2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1), DNA-directed RNA polymerase II (RPB2), 18S ribosomal RNA (18S), 28S ribosomal RNA (28S) (Table 2). Initially, this list included six more genes used as reference genes for cnidarians and other metazoans, such as eukaryotic translation factor 1 (EF1), NADH dehydrogenase iron-sulfur protein 2 ubiquinone (NADH), heat shock protein 70 (HSP70), ribosomal protein L11 (RPL11), TATA-Box Binding Protein Associated Factor 6 (TAF6), PHD finger protein 8 (PHF8). However, these genes were later excluded from the study/analysis, because either we were not able to find suitable homologues of these genes in our transcriptome/s, or primer design/PCR was not successful. The eight candidate reference genes were mined from their respective parasite transcriptome data (RNA sequences) or from DNA sequences available in GenBank or at private databases. All available homologous amino acid sequences of these genes (GenBank) from common representatives of cnidarians such as Acropora tenuis, Aurelia aurita, Hydra vulgaris, Hydra magnipapillata, Nemastella vectensis, Polypodium hydrolforme, and different myxozoan species (C. shasta, Kudoa iwatai, M. cerebralis, Sphaeroepora dicentrarchi, Thelohanellus kitauei, Buddenbrockia sp., etc.) were combined for queries. The search was performed using the tBLASTn algorithm with the e-value cutoff set to 10-5. The top hits (highest e-value) were analyzed using the NCBI conserved domains platform (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, to confirm their identity). To re-confirm the myxozoan origin of the mined sequences phylogenetic trees including other metazoan taxa (cnidarians, fish, etc.) were reconstructed using maximum likelihood methods in RAXML web-server (https://raxml-ng.vital-it.ch/#). Details on chosen sequences are included in Suppl. Mat. 1.

Primer design and specificity of PCR. Gene-specific primers were designed to amplify short 70–150 bp regions suitable for RT-qPCR assays (Table 3). Primer pairs were designed with optimal Tm at 58–60°C and GC content between 45–50%, using the NCBI online primer-design tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). All primers were tested for specificity using conventional PCR prior to performing RT-qPCR. Details on PCR conditions are described in Suppl. Mat. 5. Primer specificity was determined by obtaining single amplicons of the expected size from infected samples and no amplification in uninfected fish and worm samples (indicating that primers are not annealing with fish cDNA). Controls without reverse transcriptase (–RT) were tested for genomic DNA contamination. The presence of infection was confirmed, and parasites identified microscopically and by specific published PCR assays (indicating that primers are not annealing with fish cDNA). The identity of PCR products was confirmed by sequence comparison. RT-qPCR primer specificity was also checked by running melting curve analysis (see Suppl. Mat. 3).

Quantitative real-time PCR. RT-qPCR was performed using the LightCycler® 480 Real-Time PCR System (Roche). Reactions of 25 µl were comprised of 12.5 µl of FastStart Universal SYBR Green PCR Master Mix (Roche, Germany), 2X conc.), 1 µl of each forward and reverse primer (10 µM conc., 0.4 µM final conc.), 5 µl of PCR grade water, and 5 µl of cDNA (generally at ~150–170 ng/µl dilutions). The cycling conditions were denaturation at 95 °C for 5 min, followed by 50 cycles of 95 °C for 10 s, 58 °C for 10 s and 72 °C for 10 s. Melting curve analysis was performed after each cycle to ensure primer specificity. All samples were amplified in technical triplicates and a mean value was calculated. Four (M. cerebralis) to five (S. molnari) biological replicates were used for each sample, with the exception of C. shasta (only 3 replicates available). qPCR efficiency was predicted for each gene based on the slope of a linear regression model using a series of 5-fold dilutions (1:5, 1:25, 1:125, 1:625). Standard curves were built using Roche Light Cycler 480 Software version 1.5.0 SP4. Generally, for best amplification results efficiency ranges of 90–110% and standard curve slopes of – 3.58 to – 3.10 were considered optimal.

Ranking and quantitative analysis of reference genes. Differential expression levels and abundance of candidate reference genes within the sample was analyzed by a direct comparison of Cq (quantification cycle) values (Fig. 1, Suppl. Mat. 2). The stability of the candidate reference genes was analyzed using four algorithms: ΔCq, NormFinder, geNorm, and BestKeeper. The comparative ΔCq method manually compares relative expression of ‘pairs of genes’ within each sample. If the ΔCq value between the two genes remains constant when analyzed in different samples it means that either both genes are expressed at relatively constant rates among those samples, or they are co-regulated (here we assume the stability of both genes).
NormFinder\textsuperscript{49} was performed using original Microsoft Excel-based software. It determines the stability of the candidate genes based on an estimate of inter- and intragroup variation. It calculates the most stably expressed candidate genes and suggests two of them as references.

geNorm was performed using the qbase + package software\textsuperscript{50}. This program is based on the assumption that if the ratios between samples are uniformly expressed, non-normalized target genes should remain regular. The genes with the most irregular expression are excluded from further analysis while the last two remaining genes are selected as the most stable\textsuperscript{51}. We used two values to interpret geNorm results: (1) geNorm M (geNorm expression

| Organism           | Gene  | Primer sequence (5'-3') | Amplicon length (bp) | Melting T°(°C) | GenBank access. numbers* |
|--------------------|-------|-------------------------|----------------------|---------------|-------------------------|
| Sphaerospora molnari | ACTB  | F: AATCCACGAGACACCTCTCG 149 59.75 see Suppl. Mat. 1 |
|                    |       | R: CAGGACCGAAAACCGGGTATA 60.68 |
|                    | AHC1  | F: TTTCCCGATGTTGCTCAGAAA 138 58.94 see Suppl. Mat. 1 |
|                    |       | R: TCAATGACACCTCAGAACAGCT 59.9 |
|                    | EF2   | F: TCCGGGACAGAACAGGTGT 140 62 see Suppl. Mat. 1 |
|                    |       | R: CCAAGTTGATACGGATACGGAT 60.44 |
|                    | GAPDH | F: TATGCAGCTGCGGATTTACTGT 118 59.63 see Suppl. Mat. 1 |
|                    |       | R: GTTGTCGTCGTAATTGACCCC 59.9 |
|                    | HPRT  | F: TCTCATACGTGACCGTGCTCT 84 59.47 see Suppl. Mat. 1 |
|                    |       | R: ACGGACAAAACCGGGATCTG 59.47 |
|                    | RPB2  | F: ATTAGTTACGGTGCGAGG 143 59.54 see Suppl. Mat. 1 |
|                    |       | R: GCTGTGACATGGAAGATGCG 59.62 |
|                    | 18S rRNA | F: ATCCAGTGCTGATTCGCCTA 73 59.89 see Suppl. Mat. 1 |
|                    |       | R: ACTGCCCTGTGATGCGATT 60.32 |
|                    | 28S rRNA | F: ATCTGCTGGACACTCTACAG 143 59.97 see Suppl. Mat. 1 |
|                    |       | R: CCGGATTTGCTGCTGTTACC 60.11 |
| Myxobolus cerebralis | ACTB  | F: TTGCCTGATGGTCTAGGATG 110 59.01 AY156508.2 |
|                    |       | R: AGTGTCGTCGTAAGTCCACT 59.66 |
|                    | AHC1  | F: GTCGACGGTGCTAGAAGGA 124 59.83 GBKL01003454.1 |
|                    |       | R: GCCGGACAGACAGTCTAC 60.18 |
|                    | EF2   | F: ATGGATCAGGGCTAATCTT 149 60.7 GBKL01021688.1 |
|                    |       | R: CAAGTTCCAGCGCAACACCCC 60.6 |
|                    | GAPDH | F: GTGGCAAAAACCGCAGCTAA 95 59.61 GBKL01017634.1 |
|                    |       | R: TGTGCGGTGACAACAATGGAT 60.25 |
|                    | HPRT  | F: TGTTGCTCGTCTGAGAAAGAA 119 59.16 GBKL01050483.1 |
|                    |       | R: GAGGGTTCCATCCATCCAGTT 59.39 |
|                    | RPB2  | F: AATGGGACGCTGCTGTTAAGC 127 60.39 GBKL01027608.1 |
|                    |       | R: TAATCCCGATGTCGACAGGACC 59.53 |
|                    | 18S rRNA | F: TAGAAGTGTCGCCAACGAGTGC 85 59.48 EF370479.1 |
|                    |       | R: GGGCTCAAAGGCATCATGACA 60.03 |
|                    | 28S rRNA | F: ACGGGATAGTACGCGCTG 141 59.83 AY302740.1 |
|                    |       | R: CATCCTCAAGGATGCTGCT 59.45 |
| Ceratonova shasta | ACTB  | F: GTGGCAATTTCTCGGGTACA 149 60.04 see Suppl. Mat. 1 |
|                    |       | R: TCCAACCGGCTATTAGGAA 57.41 |
|                    | AHC1  | F: TTCCGGTTACAGCGACTCGGC 82 62.47 see Suppl. Mat. 1 |
|                    |       | R: TGAGGTGAGGGTCTAGGGA 60.55 |
|                    | EF2   | F: CGTAGTCTCCATGGGGGACCT 147 58.14 KMK392431.1 |
|                    |       | R: AAATACCTCCATGGAGCATTAGGT 57.34 |
|                    | GAPDH | F: TGGGCTCAAACAGTTGGTG 152 60.18 see Suppl. Mat. 1 |
|                    |       | R: GTGGACATTGGAAGGAGGCG 59.8 |
|                    | RPB2  | F: TGGAGGTGAGGGCACGATTGTG 156 58.58 see Suppl. Mat. 1 |
|                    |       | R: TCTGGCCCTTTTATGGAAGCG 57.54 |
|                    | 18S rRNA | F: CCGGATTGCTGCCATGCTGA 121 59.32 AF001579.1 |
|                    |       | R: CAAATTAAGCGCGAGGCTCC 59.9 |
|                    | 28S rRNA | F: ACCTGGAACGCTTAACATGGA 132 58.16 FJ981818.1 |
|                    |       | R: CCACCTGCGCCCTGAAAGATTGT 58.08 |

Table 3. Genes and their primer sequences used in this study. * Accession numbers are provided for the gene sequences that are available in GenBank/either mined from transcriptomic data under review, but see sequences in Suppl. Mat. 1.
stability value of reference genes, lowest M value indicates higher stability); and (2) geNorm V (pairwise variation). geNorm V further determines the optimal number of reference genes to be used in subsequent analyses. A \( V_{n/n+1} \) value is shown for every comparison between two consecutive numbers (n and n+1) of candidate reference genes. As a general guideline (www.qbaseplus.com, qbase+ manual, rev2017.04.27) it is stated that the benefit of using an extra (n + 1) reference gene is limited as soon as the \( V_{n/n+1} \) value drops below the 0.15 threshold, indicated with a horizontal line (Fig. 2).

BestKeeper was performed using the original Microsoft Excel-based formulas. It calculates the standard deviation of the Cq value between the whole data set, and the gene with the lowest standard deviation (SD) is proposed as most suitable.

Finally, we used RefFinder (https://www.heartcure.com.au/reffinder/?type=reference accessed at 25 June 2019), a comprehensive software platform which integrates all four algorithms providing an overall ranking of the used genes.

Transcriptomic data analyses. De novo transcriptome assemblies of *S. molnari* (unpublished) were used to observe expression of candidate reference genes in blood and sporogonic stages of parasite. We used *S. molnari* 11 samples (5 from blood stages and 6 from sporogonic stages) based transcriptomic data to estimate transcript expression values (TPM: Transcripts Per Million) using the Salmon software. These TPM expression values were scaled and served to generate a cross-sample normalized TMM gene expression matrix (TMM: trimmed mean of M-values: scaling normalization that aims to account for differences in total cellular RNA across all samples), using the Trinity package. We extrapolated TMM values for eight candidate genes expression values from the gene expression matrix and compared it across the 11 samples manually. Average values for each developmental stage were calculated. The most stable gene was considered the one for which the ratio between the average values of both developmental stages was closest to 1 (Table 4).

Results

PCR specificity and primer efficiency. Primer specificity was confirmed by obtaining single amplicons of the expected size, together with negative results in uninfected fish and worm samples. Primer specificity was also confirmed based on the occurrence of a single peak in the melting curve (Suppl. Mat. 3). Absence of genomic DNA contamination was confirmed by no amplification in –RT samples. The efficiency of our candidate RG primers in the present study ranged from 88 to 129%, which slightly surpasses the acceptable optimum range (90–110%). However, we obtained similar efficiencies for the given genes in two different developmental stages of parasite.

Cq data. Transcript abundance of each gene within each biological replicate was roughly estimated from the raw Cq values. The most abundantly expressed genes were 28S and 18S for all three studied species with Cq ranging about 14–25. The least expressed genes were AHC1 for *S. molnari* (Cq > 34) and RPB for *M. cerebralis* (Cq > 32) and *C. shasta* (Cq > 37.3). The rest of the genes fell in the range of Cq = 22–34 (Fig. 1 and Suppl. Mat. 2).
Stability of candidate genes. For *S. molnari*, the expression of EF2 and GAPDH was shown to be the most stable among the eight studied genes according to ΔCq method (lowest average SD values 1.08 for EF2 and 1.12 for GAPDH), NormFinder (with lowest stability value = 0.18 for EF2 and 0.38 for GAPDH) and geNorm (lowest M = 0.37 for both genes). We obtained pairwise variation (geNorm V-value) V2/3 < 0.15, indicating that in this case 2 genes are sufficient for normalization, and that the additional inclusion of more reference genes will not provide a significant improvement for the normalization of target genes (Fig. 2A). While GAPDH was ranked in the second most stable place (SD ± Cq = 2.15), EF2 was ranked in the third most stable place (SD ± Cq = 2) by BestKeeper (Table 5, Suppl. Mat. 4). The same genes occur in the first 4 positions across all the algorithms: geNorm: EF2 > GAPDH > ACTB > 28S, ΔCq method: EF2 > GAPDH > ACTB > 28S, NormFinder: EF2 > GAPDH > ACTB > 28S, BestKeeper: ACTB > GAPDH > 28S > EF2. To obtain a comprehensive ranking and summary of all the algorithms used, we loaded our raw data to the RefFinder web-based platform, which includes four above mentioned algorithms (Fig. 3). Overall EF2, GAPDH, and ACTB were observed as the most stable genes by RefFinder (Fig. 3A).

We observed a similar pattern for *M. cerebralis*. geNorm suggested ACTB, EF2 and GAPDH with the lowest M value (M = 0.41, 0.41 and 0.57, respectively). GeNorm pairwise variation showed middle stability, suggesting to consider 4 genes for optimal normalization (Fig. 2B). ΔCq method (with lowest average SD values 1.11 for GAPDH, 1.12 for ACTB and 1.17 for EF2) and NormFinder (with lowest stability value = 0.27 for GAPDH, 0.56 for ACTB and 0.65 for EF2) also suggested the same genes. However, BestKeeper’s ranking was different as GAPDH occurred in the fourth place (SD ± Cq = 1.69), ACTB in the fifth place (SD ± Cq = 1.95) and EF2 in the sixth place (SD ± Cq = 2). Overall, out of the eight *M. cerebralis* genes studied, the following genes ranked in the first 4 positions: geNorm: ACTB > EF2 > GAPDH > RPB2, ΔCq method: GAPDH > ACTB > EF2 > RPB2, NormFinder: GAPDH > ACTB > EF2 > RPB2, BestKeeper: 28S > 18S > AHC1 > GAPDH. RefFinder suggested GAPDH, EF2 and ACTB as the most stable reference genes in comprehensive ranking (Fig. 3B).

For *C. shasta* the combination of the genes used in the analysis was slightly different. We obtained no expression or very low expression for HPRT1 gene in all samples, and very low expression (ΔCq > 40) of RPB gene in worm samples and thus these two genes were excluded from the final gene stability analysis. geNorm showed that AHC1 and ACTB and GAPDH had the lowest M value (M = 1.27, 1.27 and 1.54 respectively). However, pairwise variation (geNormV > 0.15) could not determine the optimal number of genes to be used for normalization. (Fig. 2C). ΔCq method (with lowest average SD values 2.63 for GAPDH, 2.79 for EF2 and 2.86 for AHC1) and NormFinder (with lowest stability value = 0.94 for EF2, 1.15 for GAPDH and 1.92 AHC1) suggested EF2 and GAPDH, however BestKeeper’s ranking was different as 28S occurred in the second place (SD ± Cq = 1.97), while GAPDH and EF2 were in fourth place (SD ± Cq = 2.87), and fifth place (3.06). Overall, out of the studied *C. shasta* genes the following genes ranked in the first 4
Table 4. TMM expression values of candidate HKGs for sporogonic and blood stages of *Sphaerospora molnari*. The genes for which ratio between sporogonic and blood stages are closer to 1 are considered the most uniformly expressed between two stages.

| Genes | GeNorm | NormFinder | BestKeeper | ΔCt | Comprehensive ranking |
|-------|--------|------------|------------|-----|------------------------|
| *Sphaerospora molnari* |
| ACTB | 2 | 4 | 2 | 4 | 3 | 4 |
| AHC1 | 5 | 7 | 7 | 6 | 6 | 8 |
| EF2 | 1 | 1 | 5 | 1 | 1 | 1 |
| GAPDH | 1 | 2 | 3 | 2 | 2 | 2 |
| HPRT1 | 4 | 5 | 6 | 5 | 7 | 7 |
| RPB2 | 7 | 8 | 8 | 8 | 8 | 3 |
| 18S rRNA | 6 | 6 | 4 | 7 | 5 | 5 |
| 28S rRNA | 3 | 3 | 1 | 3 | 4 | 6 |
| *Myxobolus cerebralis* |
| ACTB | 1 | 2 | 5 | 2 | 2 | 2 |
| AHC1 | 5 | 5 | 3 | 6 | 6 | 6 |
| EF2 | 1 | 3 | 6 | 3 | 3 | 3 |
| GAPDH | 2 | 1 | 4 | 1 | 1 | 1 |
| HPRT1 | 4 | 6 | 8 | 5 | 8 | 8 |
| RPB2 | 3 | 4 | 7 | 4 | 5 | 5 |
| 18S rRNA | 7 | 8 | 2 | 8 | 7 | 7 |
| 28S rRNA | 6 | 7 | 1 | 3 | 4 | 4 |
| *Ceratonia shasta* |
| ACTB | 1 | 4 | 5 | 4 | 6 | 6 |
| AHC1 | 2 | 3 | 4 | 3 | 3 | 3 |
| EF2 | 4 | 1 | 3 | 2 | 2 | 2 |
| GAPDH | 3 | 2 | 2 | 1 | 1 | 1 |
| HPRT1 | Excluded from analysis | | | | | |
| RPB2 | Excluded from analysis | | | | | |
| 18S rRNA | 8 | 6 | 6 | 6 | 6 | 5 |
| 28S rRNA | 7 | 4 | 1 | 5 | 4 | 4 |

Table 5. Comprehensive ranking of studied genes using a combination of four algorithms. TMM ranking is based on normalized transcript expression values from NGS data. Lower ranking values indicate higher gene stability. Additionally, gene stability values generated by each algorithm are given in Suppl. Mat. 4.

positions: geNorm: AHC1 > ACTB > GAPDH > EF2, ΔCq: GADH > EF2 > AHC1 > ACTB, NormFinder: EF2 > GAPDH > AHC1 > ACTB, BestKeeper: 28S > GAPDH, EF2 > AHC1 (Table 5). RefFinder suggested EF2, GAPDH and AHC1 as the most stable reference genes in comprehensive ranking (Fig. 3C).
Differential gene expression from transcriptome data. We have obtained TMM values for candidate reference genes in two *S. molnari* developmental stages (pre-sporogonic blood stage and sporogonic stage). We have calculated the closest to 1 value for EF2 (ratio between two stages = 0.94) and GAPDH (ratio between two stages = 0.91) indicating these two genes as the most stable of the eight studied genes (see Tables 4 and 5).

**Discussion**

Expectations for real-time RT-qPCR are high as it serves as a first step of generating a data which will be a reference for the next steps of research applications (i.e. studies involving gene expression data).

While, numerous studies are based on qPCR data, in the past, only few reference gene validation studies were conducted. Many papers would use only a single gene as a reference, without verification of its utility under the used experimental conditions. This was especially common for majority of the articles concerning the analysis of RNA transcripts published in high impact journals in late 1990s and early 2000s in different organisms including myxozoans.

In order to obtain reliable results, reference genes normalization and its rational interpretation are essential. It is complicated to determine if a two-fold variation in gene expression is of biological importance because this genetic variation can be triggered by intrinsic noise of biochemical reactions. Discrepancies with regard to organism strains, experimental design, and algorithms calculating differential expression further add to this noise.

While not everything can be controlled for, the first step for producing meaningful (true) data is careful evaluation of reference genes.

Since previous data evaluating the stability of reference genes in myxozoans are missing, in this study, we evaluated eight candidate genes for their suitability as a reference for future RT-qPCR assays in gene expression studies of myxozoans. We designed a comprehensive setup for testing these genes in a comparative approach by using RNA extractions from different developmental stages of the parasites’ dixenous life cycle, using three species from different phylogenetic lineages, covering the fields of biological and technical replicates and different calculation algorithms and methods by using RT-qPCR and transcriptomic data. Here, we discuss the parameters we used to ensure the best choice of reference genes, possible pitfalls that should be taken into consideration before final conclusions, and we provide recommendations for future RT-qPCR studies in this unique group of highly derived cnidarian parasites.

**Stability of the candidate reference gene and choice of algorithm.** We used four well-accepted algorithms, geNorm, ΔCq, NormFinder, and BestKeeper in combination with TMM expression values mined in transcriptome expression data to evaluate the stability of the examined genes. Since these algorithms have
different calculating approach, it might expect that the rankings of candidate genes could be different depending on the software applied. In previous studies we observed that the results produced by BestKeeper can oppose those of geNorm and NormFinder. Each approach has its strengths and weaknesses and there is no commonly accepted opinion on which one is the best. A consensus ranking of RGs is useful as it combines the data obtained from different algorithms and creates a meaningful outcome reflecting an overall agreement. We used RefFinder for consensus ranking. We additionally checked the expression levels of examined genes in 6 transcriptomic datasets of highly proliferative and motile feeding stages vs 5 localized, predominantly intracellular spore-forming stages, for further confirmation of qPCR data obtained. In principle, RNASeq data can be used to identify good reference genes without previous selection according to published data. This is offering attractive perspectives regarding new RG discoveries, since the proposed workflows can be used for already generated transcriptomic datasets, regardless of sequencing technology, library size or organism. With regard to myxozoans, there are not enough transcriptomic datasets of different developmental stages available for a single species and we hence compared preselected genes in new transcriptomic data of two different developmental stages of *S. molnari*. While other studies used TPM (Transcripts Per Million) or FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values from RNA-Seq studies to analyse gene expression stability via CV (coefficient of variation) and fold change calculation methods we used a relatively straightforward approach, simply comparing already cross-sample normalized TMM values of the gene between studied conditions.

### Stability of the candidate reference genes and experimental conditions

Numerous studies showed that the stability of proposed HKGs vary across organisms and most importantly experimental conditions (i.e. developmental stages, drug/dietary treatment, temperature such as heat shock, cold stress, drought stress, etc.). Only genes that have stable expression under a condition to be analysed can be used as an RG for the given study. In the present case, we were mainly focused on providing RGs that are stable during myxosporean development, rather than e.g. under different temperature or dietary regimes. This is of particular importance to be able to investigate the stage-specific expression profile of parasite genes.

In our study, comprehensive ranking together with transcriptomic TMM calculations suggested that EF2 and GAPDH are the most stable genes across all the studied myxozoan species (Table 5). EF2 promotes the GTP-dependent translocation of the ribosome. It is an essential factor for protein synthesis and thus, like other ribosomal genes, is assumed to have a constant expression throughout different tissues, different treatments or developmental stages of the organism. It has been shown to be the most stable gene for mouse DNBS disease treated and non-treated colon tissue, or for plant tissues exposed to biotic and abiotic stress. GAPDH is one of the most used RGs in different organisms, including corals, fish, human, etc. Although several studies showed that GAPDH can be regulated under some experimental conditions, i.e. gene expression in thermal and light studies it is reported to be a suitable reference for normalizing gene expression in various life stages, for instance in red algae, plants or during the metamorphosis of free living cnidarian representatives such as corals (*Porites astreoides*), which coincides well with the general scheme of our study. Our results suggested GAPDH as the first most stable gene for *M. cerebralis* and *C. shasta*, and the second most stable gene for *S. molnari*.

Actin is one of the most conserved proteins in eukaryotes, whose structure has been conserved despite the numerous actin isoforms reported with different biological functions. Beta actin is one of the most common reference genes used in gene expression studies as it is known to be a key component involved in the development of cytoskeletal filaments. It was listed as one of the best-performing reference genes in cnidarian/dinoflagellate studies. Similarly, it was the best performing reference gene, along with 28S rRNA, in the parasitic apicomplexan *Theileria parva* from different host tissues. Beta actin was used as a reference gene for *M. cerebralis* and a number of gene expression studies in free-living cnidarians, in various experimental conditions and was redefined as an unsuitable reference gene in some cases. Multiple actin isoforms are known from myxozoans and in *S. molnari*, the expression level of two highly divergent isoforms differs about 15-fold, since likely only isoform 1 is responsible for the unique parasite motility during proliferation in the blood. These highly variable expression levels of different isoforms suggest different functions and even though we used actin isoform 2, which was expressed at a low level, for the design of our qPCR assay, our results did not support beta actin to be an optimal reference gene for *S. molnari* or the other myxozoan species studied. It was ranked only in third place for *S. molnari* and *M. cerebralis*, and in sixth for *C. shasta*. TMM data also placed it in the fourth place for *S. molnari*. Actin isoforms are very similar to beta actin and gamma actin, differing only by four biochemically similar residues and being conserved from birds to mammals, however, actins show highly divergent DNA sequences in myxozoans. Hence, the possibility of misidentification of the same actin isoform in different species of the highly derived myxozoans gives an additional reason for excluding beta actin from qPCR analyses in myxozoans.

Alongside with beta actin, ribosomal genes especially 18S and 28S rRNA are traditionally used as reference genes. Being structural components of small and large eukaryotic ribosomal subunits (40S and 60S), they are one of the most basic components of eukaryotic cells. However, the suitability of 18S and 28S rRNA as a reference gene varies in the literature. In myxozoan studies 18S was used for phylogenetic studies and for detection and quantification of parasites in environment or host tissue. Thus, we have included it in our study to test its utility as reference gene, since it is shown to have high stability. However, despite its high stability in a number of organisms many studies indicated that these are highly expressed genes and are often unsuitable for comparison. It can be challenging to compare them with target genes expressed at a low level which can lead to erroneous results. Indeed, 18S and 28S rRNA were the most highly expressed genes (Cq < 21) in our study, except for *C. shasta* (Cq for 18S = 23.9–25.6 in intestine and worm samples, respectively). In our study 28S was the fourth most stable gene for *S. molnari* and *C. shasta*.
Stability of the candidate reference genes and the influence of used methodology. Despite RT-qPCR being one of the most reliable techniques to accurately measure the expression level of a gene, there are number of factors that may affect the consistency of expression data. For instance, different dyes can influence PCR inhibition in a concentration-dependent manner and can have effects on DNA melting temperature or can preferentially bind to certain DNA sequences81. PCR efficiency can be influenced by PCR inhibitors present in the sample or by non-optimal primer design. This information is critical, since these factors can produce different results even if the experimental design or study organism is similar (see also notes in Suppl. Mat. 5). Overall, we obtained consistent results for the suitability of RGs in three myxosporean species from different phylogenetic clades: EF2, GAPDH together with ACTB were the most stable genes for S. molnari and M. cerebralis, and EF2, GAPDH and AHC1 for C. shasta. Additionally, comparable TMM values of S. molnari demonstrate the robustness of our predictions. Thus, it can be hypothesized that these genes will also have stable expression in other related myxozoan species. These results are useful in particular for studies involving developmental stages of these parasites, however furthermore it would be of great interest to check the stability of these genes under different experimental conditions such as temperature, drug treatment, different dietary treatment, water quality, etc.

Possible pitfalls of detecting less abundant transcripts and further recommendations. Likewise, highly expressed genes, such as 18S and 28S rRNA, reference gene may not be suitable to use in gene expression studies, if the gene of interest (i.e. target gene) has low expression in comparison to the reference gene. For instance, in our study, suggested reference genes (EF2, GAPDH) showed a suitable expression range (Cq = 23–33) in all samples (see Fig. 1, and Suppl. Mat. 2 for the abundance of each gene in each sample). However, we noticed that all the investigated genes in these samples showed low expression levels, which could be related to the low amount of parasite concentration in the sample or PCR inhibition, which may occur in guts and soil samples71,82–84.

PCR inhibition is something to be aware of, especially in invertebrate host extractions procedures, and need to be carefully evaluated to ensure sufficient representation of quantifiable transcripts in subsequent myxozoan studies.

Several attempts (i.e. using inhibitor removal column and reagents, see details in Suppl. Mat. 5) were undertaken to reduce inhibitory effect in this study, which did not improve our overall results. Another useful way to evaluate the inhibitory effect is to use serial dilutions, since inhibitory effect can be lost in high dilutions. However, for the samples where the parasite concentration is already extremely low, dilution may not always be an optimal solution.

While we cannot exclude PCR inhibition as the reason for low detection of some of our genes, it is possible that the high Cq values observed are simply related to low parasite concentrations in the sample or these transcripts are expressed in low levels.

In either way, detecting less abundant transcripts remain an open question, especially in invertebrate samples, and more analyses (i.e. using new inhibitory effects removal kits, testing different concentration of RNA, carefully evaluating the primer design) may help to clarify this problem.

Finally, using a single reference gene for gene normalization is generally less reliable than the use of a set of genes85, and based on our data, we propose using a combination of at least 2 to 3 genes for myxozoans. To our knowledge, this is the first study to validate RGs for myxozoan species, and we are convinced that the results presented here serve as an essential aid for subsequent gene expression studies of this group of extremely derived parasites.

Data availability
The data that support the findings of this study are available in Supplementary Material.

Received: 13 January 2019; Accepted: 2 October 2019;
Published online: 21 October 2019

References
1. Wolf, K. & Markiw, M. E. Biology contravenes taxonomy in the Myxozoa: New discoveries show alternation of invertebrate and vertebrate hosts. Science 225, 1449–1452 (1984).
2. Exterbauer, E. et al. Myxozoan life cycles: Practical approaches and insights. In Myxozoan Evolution, Ecology and Development 175–198, https://doi.org/10.1007/978-3-319-14753-6_10 (Springer International Publishing, 2015).
3. Kent, M. L. et al. Recent advances in our knowledge of the Myxozoa. J. Eukaryot. Microbiol. 48, 395–413 (2001).
4. Sterud, E. et al. Extreme longevity in wild Atlantic salmon Salmo salar due to proliferative kidney disease (PKD) caused by Tetracapsuloides bryosalmonae (Myxozoa). Dis. Aquat. Organ. 77, 191–198 (2007).
5. Peeler, E. J. & Taylor, N. G. The application of epidemiology in aquatic animal health – opportunities and challenges. Vet. Res. 42, 94 (2011).
6. Hallett, S. L. & Bartholomew, J. L. Myxobolus cerebralis and Ceratomyxa shasta. In Fish parasites: pathobiology and protection (eds Woo, P. T. K. & Buchmann, K.), 141–172 ((CABI), 2012).
7. True, K., Voss, A. & Foott, J. S. Myxosporean parasite (Ceratomyxa shasta and Parvicapsula microbicornis) prevalence of infection in Klamath river basin juvenile Chinook salmon, March–August 2017. Calif. - Nevada Fish Heal. Cent. (2013).
8. Fontes, I., Hallett, S. L. & Mo, T. A. Comparative epidemiology of myxozoan diseases. In Myxozoan Evolution, Ecology and Development. (eds Okamura, B. & et al) 85–110 (Springer Int. Pub, 2015).
9. FAO. The State of world fisheries and aquaculture 2016. Contributing to food security and nutrition for all, Rome, 200 pp (2016).
10. FAO. Aquaculture newsletters. No 56 Rome, 51pp (2017).
11. Clifton-Hadley, R. S., Bucke, D. & Richards, R. H. Economic importance of proliferative kidney disease in salmonid fish in England and Wales. Vet. Res. 119, 305–306 (1986).
12. Granath, W. O., Gilbert, M. A., Wyatt-Pescador, E. J. & Vincent, E. R. Epizootiology of Myxobolus cerebralis, the causative agent of salmonid whirling disease in the rock creek of west-central Montana. J. Parasitol. 93, 104–119 (2007).
13. Ray, R. A., Holt, R. A. & Bartholomew, J. L. Relationship between temperature and Ceratomyxa shasta–induced mortality in Klamath river salmonids. J. Parasitol. 98, 520–526 (2012).
41. Ray, R. A., Alexander, J. D., De Leenheer, P. & Bartholomew, J. L. Modeling the effects of climate change on disease severity: A case study of Ceratoneura (syn Ceratomyxa) shasta in the Klamath river. In Myxozoan Evolution, Ecology and Development 363–378, https://doi.org/10.1007/978-3-319-14753-6_19 (Springer International Publishing, 2015).

42. Holzer, A. S. et al. The joint evolution of the Myxozoa and their alternate hosts: A cnidarian recipe for success and vast biodiversity. Mol. Ecol. 27, 1651–1666 (2018).

43. Chang, E. S. et al. Genomic insights into the evolutionary origin of Myxozoa within Cnidaria. Proc. Natl. Acad. Sci. 112, 14912–14917 (2015).

44. Molnár, K. Gill sphaerosporosis in the common carp and grass carp. Acta. Vet. Acad. Sci. Hungary 27, 99–113 (1979).

45. Korytã, T. et al. The kinetics of cellular and humoral immune responses of common carp to presporogenic development of the myxozoan Sphaerospora molnari. Parasites & Vectors 12, 208 (2019).

46. Hellemans, J., Mortier, G., De Paepe, A., Speleman, F. & Vandesompele, J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8, R19 (2007).

47. Chong, G., Kuo, F.-W., Tsai, S. & Lin, C. Validation of reference genes for cryopreservation studies with the gorgonian coral Acropora millepora. J. Fish Dis. 36, 303–314 (2012).

48. Atkinson, S. D. & Bartholomew, J. L. Disparate infection patterns of Myxobolus spp. (Myxozoa) in fish blood. Anal. Biochem. 162, 156–159 (1987).

49. Eszterbauer, E. et al. Molecular characterization of Sphaerospora molnari (Myxozoa), the agent of gill sphaerosporosis in common carp Cyprinus carpio carpio. Dis Aquat Organ. 104(1), 59–67 (2013).

50. Atkinson, S. D., Hallett, S. L. & Bartholomew, J. L. Genotyping of individual Ceratonyxova shasta (Cnidaria: Myxozoa) myxospores reveals intra-spore ITS-1 variation and invalidates the distinction of genotypes II and III. Parasitol. 145, 1588–1593 (2018).

51. Pfaff, M. W. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45 (2001).

52. Silver, N., Best, S., Jiang, J. & Thein, S. L. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time quantitative RT-PCR. BMC Mol. Biol. 7, 33 (2006).

53. Andersen, C. L., Jensen, J. L. & Ornston, T. F. Normalization of Real-Time Quantitative Reverse Transcription–PCR Data: A Model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Genet. Cytogenet. 64, 5245–5250 (2004).

54. Holzer, A. S., Molnár, K. & Jorgensen, A. The joint evolution of the Myxozoa and their alternate hosts: A cnidarian recipe for success and vast biodiversity. Mol. Ecol. 27, 1651–1666 (2018).

55. Chang, E. S. et al. Genomic insights into the evolutionary origin of Myxozoa within Cnidaria. Proc. Natl. Acad. Sci. 112, 14912–14917 (2015).

56. Molnár, K. Gill sphaerosporosis in the common carp and grass carp. Acta. Vet. Acad. Sci. Hungary 27, 99–113 (1979).

57. Korytã, T. et al. The kinetics of cellular and humoral immune responses of common carp to presporogenic development of the myxozoan Sphaerospora molnari. Parasites & Vectors 12, 208 (2019).

58. Hellemans, J., Mortier, G., De Paepe, A., Speleman, F. & Vandesompele, J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8, R19 (2007).

59. Vandesompele, J. et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, research0034.1 (2002).

60. Pfaff, M. W., Tichopad, A., Prigomet, C. & Neuvians, T. P. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. Biotechnol. Lett. 26, 509–15 (2004).
53. Patro, R., Duggal, G., Love, M. I., Irizarry, R. A. & Kingsford, C. Salmon provides fast and bias-aware quantification of transcript expression. *Nat. Methods* **14**, 417–419 (2017).
54. Robinson, M. D. & Oshlack, A. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol.* **11**, R25 (2010).
55. Haas, B. J. et al. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat. Protoc.* **8**, 1494–1512 (2013).
56. Hribal, S., Scharsack, J. P. & Becker, S. Housekeeping genes for quantitative expression studies in the three-spined stickleback *Gasterosteus aculeatus*. *BMC Mol. Biol.* **9**, 18 (2008).
57. Dzaki, N., Ramli, K. N., Azlan, A., Ishak, I. H. & Azzam, G. Evaluation of reference genes at different developmental stages for quantitative real-time PCR in *Aedes aegypti*. *Sci. Rep.* **7**, 43618 (2017).
58. Pflüh, V., Datta, S. & Datta, S. RankAffqreg, an R package for weighted rank aggregation. *BMC Bioinformatics* **10**, 62 (2009).
59. Vanhauwaert, S. et al. Expression repeat elements improve RT-qPCR normalization across a wide range of zebrafish gene expression studies. *PLoS One* **9**, e109091 (2014).
60. Carmona, R. et al. Automated identification of reference genes based on RNA-seq data. *Biomed. Eng. Online* **16**, 65 (2017).
61. Gao, D., Kong, F., Sun, P., Bi, G. & Yao, Y. Transcriptome-wide identification of optimal reference genes for expression analysis of *Pyropia yezoensis* responses to abiotic stress. *BMC Genomics* **19**, 251 (2018).
62. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–140 (2010).
63. Stanton, K. A. et al. A Whole–transcriptome approach to evaluating reference genes for quantitative gene expression studies: A case study in *Mimulus G.* (Bethesda) 7, 1085–1095 (2017).
64. Brunner, A. M., Yakovlev, I. A. & Strauss, S. H. Validating internal controls for quantitative plant gene expression studies. *BMC Plant Biol.* **4**, 14 (2004).
65. de Jonge, H. J. M. et al. Evidence based selection of housekeeping genes. *PLoS One* **2**, e898 (2007).
66. Susarov, D. et al. Eukaryotic translation elongation factor 2 (eEF2) catalyzes reverse translocation of the eukaryotic ribosome. *J. Biol. Chem.* **293**, 5220–5229 (2018).
67. Essa, N. et al. Stability of reference genes for messenger RNA quantification by Real-Time PCR in mouse dextran sodium sulfate experimental colitis. *PLoS One* **11**, e0156289 (2016).
68. Boava, L. P. et al. Selection of endogenous genes for gene expression studies in *Eucalyptus* under biotic (*Puccinia psidii*) and abiotic (acibenzolar-S-methyl) stresses using RT-qPCR. *BMC Res. Notes* **3**, 43 (2010).
69. Zainuddin, A., Chua, K. H., Abdul Rahim, N. & Makpol, S. Effect of experimental treatment on GAPDH mRNA expression as a housekeeping gene in human diploid fibroblasts. *BMC Mol. Biol.* **11**, 39 (2010).
70. Dombrowski, J. E. & Martin, R. C. Evaluation of reference genes for quantitative RT-PCR in *Lolium temulentum* under abiotic stress. *Plant Sci.* **176**, 390–396 (2009).
71. Lovdal, T. & Liljo, C. Reference gene selection for quantitative real-time PCR normalization in tomato subjected to nitrogen, cold, and light stress. *Anal. Biochem.* **387**, 238–242 (2009).
72. Wu, X. et al. Variation of expression levels of seven housekeeping genes at different life-history stages in *Porphyra yezoensis*. *PLoS One* **8**, e60740 (2013).
73. Li, M.-Y. et al. Validation and comparison of reference genes for qPCR normalization of *Celery* (*Apium graveolens*) at different development stages. *Front. Plant Sci.* **7**, 313 (2016).
74. Kelley, G. O., Beauchamp, K. A. & Hedric, R. Phylogentic comparison of the myxosporea based on an actin cDNA isolated from *Myxobolus cerebralis*. *J. Eukaryot. Microbiol.* **51**, 660–663 (2004).
75. Hartigan, A. et al. New cell motility model observed in parasitic cnidian *Sphaerospora molnari* (*Myxozoa: Myxosporea*) blood stages in fish. *Sci. Rep.* **6**, 39093 (2016).
76. Perrin, B. J. & Ervasti, J. M. The actin gene family: function follows isoform. *Cytoskeleton* **67**, 630–634 (2010).
77. Kim, B.-R., Nam, H.-Y., Kim, S.-U., Kim, S.-I. & Chang, Y.-J. Normalization of reverse transcription quantitative-PCR with housekeeping genes in *rice*. *Biotechnol. Lett.* **25**, 1869–72 (2003).
78. Huggert, J., Dheda, K., Bastin, S. & Zamula, A. Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun.* **6**, 279–284 (2005).
79. Kuchipudi, S. V. et al. 18S rRNA is a reliable normalisation gene for real time PCR based on influenza virus infected cells. *Virol. J.* **9**, 230 (2012).
80. Bar, M., Bar, D. & Lehmann, B. Selection and validation of candidate housekeeping genes for studies of human Keratinocytes— Review and recommendations. *J. Invest. Dermatol.* **129**, 535–537 (2009).
81. Gudnason, H., Dufla, M., Bang, D. D. & Wolff, A. Comparison of multiple DNA dyes for real-time PCR: effects of dye concentration and sequence composition on DNA amplification and melting temperature. *Nucleic Acids Res.* **35**, e127 (2007).
82. Penn, H. J., Chapman, E. G. & Harwood, J. D. Overcoming PCR inhibition during DNA-based gut content analysis of ants. *Environ. Entomol.* **45**, 1255–1261 (2016).
83. Schrader, C., Schielke, A., Eckerle, L. & Johne, R. PCR inhibitors—Occurrence, properties and removal. *J. Appl. Microbiol.* **113**, 1014–1026 (2012).
84. Matheson, C. D., Gurney, C., Esau, N. & Lehto, R. Assessing PCR Inhibition from humic substances. *Open Enzym. Inhib.* **3**, 38–45 (2014).
85. Busin, S. A. et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin. Chem.* **55**, 611–622 (2009).
86. NajafiPanah, M. J., Sadeghi, M. & Bakhtiarizadeh, M. R. Reference genes selection for Quantitative Real-Time PCR using RankAggreg method in different tissues of *Capra hircus*. *PLoS One* **8**, e83041 (2013).
87. Radonic, A. et al. Guideline to reference gene selection for quantitative real-time PCR. *Biochem. Biophys. Res. Commun.* **333**, 856–862 (2004).
88. Stürenbaum, S. R. & Kille, P. Control genes in quantitative molecular biological techniques: the variability of invariance. *Comp. Biochem. Physiol. Part B* **130**, 281–289 (2001).
89. Tsotsi, T. N., Collins, N. E., Oosthuizen, M. C. & Sibeeko-Matjila, K. P. Selection and evaluation of housekeeping genes as endogenous controls for quantification of mRNA transcripts in *Theileria parva* using quantitative real-time polymerase chain reaction (qPCR). *PLoS One* **13**, e0196715 (2018).

**Acknowledgements**

We acknowledge financial support by the Czech Science Foundation (grant numbers 19–28399X (to AK) and 14–28784P (to GAB)), the European Commission (project reference 634429, ParaFishControl (to ASH)), the Ministry of Education, Youth and Sports of the Czech Republic (project no. ITUSA17201 (to PBS)), the Hungarian National Research, Development and Innovation Office (project no. NN124220 (to EE)) and Consellería de Educación, Investigación, Cultura y Deporte, Valencia, Spain (APOSTD/2013/087 (to GAB)). We thank Stephen D. Atkinson and Julie Alexander (OSU, USA) for the help in Manayunkia worm sampling.
Author contributions
A.K. and A.S.H. conceived and designed the study. A.S.H., G.A.B., P.B.S., A.B.T., E.E. and J.B. collected/provided the scientific material. A.K., A.S.H., R.S. and A.N. design the methodology. A.K, G.A.B. and P.B.S. generate the data. A.K. analyzed the data. All authors contributed for writing and approving the final manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-019-51479-0.

Correspondence and requests for materials should be addressed to A.K.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019