Comparative Biochemistry and Physiology, Part B 151 (2008) 296–304

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Stability of reference genes for real-time PCR analyses in channel catfish (*Ictalurus punctatus*) tissues under varying physiological conditions

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ARTICLE INFO

Article history:
Received 23 April 2008
Received in revised form 14 July 2008
Accepted 17 July 2008
Available online 22 July 2008

Keywords:
Channel catfish
Housekeeping genes
Normalization
Real-time PCR

ABSTRACT

Real-time PCR is a highly sensitive, relatively easy to perform assay for quantifying mRNA abundance. However, there are several complexities built into the assay that can affect data interpretation. Most notably, the selection of an appropriate internal control for normalization is essential for expression data interpretation. In this study we investigated the suitability of seven commonly used genes [18S ribosomal RNA (18S), alpha tubulin (TUBA), beta actin (ACTB), beta-2 microglobulin (B2M), embryonic elongation factor-1 alpha (EEF1A), glyceraldehyde phosphate dehydrogenase (GAPDH), and RNA polymerase II polypeptide B (POLR2B)] as potential quantitative references for normalizing real-time PCR data generated in the study of channel catfish physiology. Gene expression and stability were evaluated among 15 channel catfish tissues and within physiologically-relevant tissues in response to experimental manipulation (i.e. LHRH injection, fasting, and acute stress). Expression of the seven candidate reference genes varied across all tissue types tested, indicating that none of the genes could suitably serve as reference genes for cross tissue comparisons. Experimentally altering the physiological state of the fish differentially affected expression of the various reference genes depending on experimental design and tissue type, with 18S unaffected by the experimental treatment in all tissues examined. For example, the selection of a differentially expressed gene, GAPDH, as opposed to 18S, to normalize hepatic growth hormone receptor during fasting resulted in misinterpretation of the data. These results reveal the importance of providing comprehensive details of reference gene validation when publishing real-time PCR results, with this manuscript serving as a basic guideline for reference gene selection in channel catfish research.

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1. Introduction

Elucidation of the molecular mechanisms regulating the physiology, biochemistry, and endocrinology of fish has been slow relative to that of domestic mammals used for research and agriculture. Recently, however, fish genome sequencing projects, such as the Genomic Research on Atlantic Salmon (GRASP; http://webuvic.ca/cbr/grasp/) and the Salmon Genome Project (SGP; http://www.salmongenome.no/cgi-bin/sgp.cgi) have made available large numbers of expressed sequence tags (ESTs) that are quite valuable to fish physiologists. By mining these and similar databases, researchers have been able to garner sequence information for candidate genes utilized in gene expression assays (e.g. real-time PCR and microarrays).

Quantitative real-time PCR represents a highly sensitive, reproducible, and high throughput methodology for measuring the relative abundance of mRNA transcripts from a given sample. This particular methodology is attractive when compared to other options for measuring gene expression at the transcriptional level, especially when considering the time consuming procedures of quantitative northern blot analysis, RNase protection assays, and quantitative competitive PCR. With all of the aforementioned methods (including real-time PCR), relative expression levels need to be normalized to an internal standard before any meaningful comparisons of expression levels can be made between different tissues/cell lines. Normalization is essential for reduction of sample to sample variation that may occur at multiple steps in the quantification process (i.e., RNA extraction, cDNA synthesis, PCR amplification, etc.). The selection of an internal standard presents itself with two basic options. First, one may utilize an exogenous internal standard with the option of adding it to the sample at any one of the number of steps in the quantification process (e.g. adding the internal standard to the tissue sample prior to RNA isolation). Additionally, these exogenous internal standards may be designed to utilize the same primer set as that of the particular gene of interest, thereby reducing variability that may occur in amplification

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1096-4959/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.cbpb.2008.07.010

efficiency. The second option is to utilize an endogenous internal standard concurrently expressed with the gene of interest in the tissues/cell lines. Using this option, one assumes the endogenous internal standard is expressed in a constitutive manner with no significant variation between samples.

An increasing number of studies have utilized quantitative real-time PCR for measuring gene expression in various species of fish, including channel catfish, *Ictalurus punctatus*. The availability of large numbers of channel catfish expressed sequence tags (ESTs) in public databases, such as Genbank dbEST (http://www.ncbi.nih.gov) and the Catfish Gene Index (http://compbio.dfci.harvard.edu/tgi/tgipage.html), has stimulated studies of functional gene expression to define molecular regulation of physiological pathways in this species. For example, a number of studies on growth (Peterson et al., 2004; Clay et al., 2005; Peterson and Small 2005; Small and Peterson 2005; Kaiya et al., 2005; Small et al., 2006), immune response (Bilodeau and Waldbieser 2005; Bilodeau et al., 2006; Peterson et al., 2007; Bilodeau-Bourgeois et al., 2008), and stress (Karsi et al., 2004, 2005; Weber and Bosworth, 2005) in channel catfish have been reported using expression analysis with real-time PCR. In these studies, and in fact most reported studies using real-time PCR methodologies, normalization of expression data was conducted using a single, endogenous, simultaneously expressed reference gene as an internal standard. When using endogenous reference genes for normalization, it is assumed that expression levels for the reference gene do not vary significantly between tissues or treatment groups (e.g., between disease states or physiological treatment groups). However, recent data with a variety of species suggests that many commonly used reference genes, such as the actins and GAPDH, appear to vary significantly depending on the developmental or physiological state (Thellin et al., 1999; Bustin, 2000; Jorgensen et al., 2006; Fernandes et al., 2008). The realization that these reference genes, commonly used in less sensitive assays of gene expression (e.g. northern blot analysis), are not always suitable for real-time PCR has led experienced researchers to declare the necessity of validating reference genes for each experimental situation (Vandesompele et al., 2002; Dheda et al., 2004). Others have advocated normalization of samples against cell number, rRNA or total RNA (Bustin 2000, 2002). In fact, common practice for most authors has been to provide minimal or no validation of their normalization procedures, which raises serious concerns about the validity of any conclusions related to gene expression levels.

Real-time PCR can be conducted with relative ease and rapidity, and has become a staple analytical tool for biologists to interpret the responsive and regulatory effects of genes *in vitro* and *in vivo*. In this study we report the analysis of seven routinely used reference genes including: 18S ribosomal RNA (18S), alpha tubulin (TUBA), beta actin (ACTB), beta-2 microglobulin (B2M), embryonic elongation factor-1 alpha (EEF1A), glyceraldehyde phosphate dehydrogenase (GAPDH), and RNA polymerase II polypeptide B (POLR2B). Expression levels of all seven potential reference genes were analyzed among 15 channel catfish tissues and within specific tissues in response to experimentally induced changes in physiological state. First, the effects of exogenous luteinizing hormone-releasing hormone (LHRH) treatment on the expression of all seven reference genes in the hypothalamic–pituitary–gonadal axis were addressed. Second, the potential effects of food deprivation on the expression patterns of these reference genes were quantified in tissues within the somatotropic axis. Lastly, patterns of expression of these reference genes were compared in tissues within the hypothalamic–pituitary–interrenal “stress” axis in response to an acute low-water stress event. The objective was to determine how tissue type and experimentally-altered physiological status affect the stability of potential reference gene expression and interpretation of target gene response. The effect of using the “wrong” reference gene on interpreting hepatic gene expression data from fasted catfish is illustrated.

2. Materials and methods

2.1. Channel catfish

All channel catfish described in the following experiments were of the NWAC103 commercial strain maintained at the USDA-ARS Catfish Genetics Research Unit, Stoneville, MS following accepted standards of animal care and use approved by the Institutional Animal Care and Use Committee (IACUC) according to USDA-ARS policies and procedures.

2.2. Tissue expression

Hypothalamus, pituitary, gill, muscle, stomach, intestine, heart, liver, spleen, gall bladder, Brockman bodies, head kidney, trunk kidney, and ovary tissues were dissected from four reproductively mature female channel catfish and testis tissue from four reproductively mature male channel catfish, placed into individual 2-mL cryogenic tubes filled with 1 mL of TriReagent (Molecular Research Center, Inc., Cincinnati, OH, USA), and flash frozen in liquid nitrogen. All tissue samples were then stored at −80 °C until RNA isolation.

2.3. LHRHa treatments

The effect of LHRHa injection on reference gene expression was investigated in reproductively mature, 3-yr-old, female channel catfish. Three channel catfish females received intraperitoneal injections of LHRH analog (Sigma-Aldrich, St. Louis, MO) at a dosage of 100 μg/kg body mass. Three additional female channel catfish were injected with phosphate buffer solution (1× PBS) to serve as a control group. Initial blood samples were collected from the caudal vasculature of all six fish prior to the injections. Following the injections, all fish were maintained in a concrete raceway until 12-h post-injection, then euthanized in a 300-mg/L solution of tricaine methanesulfonate (MS-222; Argent Laboratories, Redmond, WA, USA), followed by tissue sample collection from all six fish. Tissue samples included blood, hypothalamus, pituitary, ovary, and liver. Blood samples were held in ice until separation of plasma. The remaining tissues were placed immediately into individual 2-mL cryogenic tubes filled with 1 mL of TriReagent (Molecular Research Center, Inc.) and flash frozen in liquid nitrogen. All tissue samples were stored at −80 °C until processed.

Blood samples (1 mL) were centrifuged for 5 min at 1000 *g* to facilitate the separation of plasma. Plasma samples were collected and then stored at −80 °C. 17β-estradiol assays were conducted using an enzyme-linked immunosorbent assay (ELISA) procedure (Oxford Biomedical Research, Oxford, MI, USA). Intra- and inter-assay coefficients of variation were less than 10%. Accuracy, calculated as the percent of exogenous estradiol recovered from spiked fish plasma, was greater than 95%. Steroids were extracted from plasma samples (100 μL) with 1 mL of ethyl ether and then incubated with 50 μL of estradiol–horseradish peroxidase conjugate in antibody coated plates for 1 h at room temperature. Bound enzyme conjugate was detected by addition of 150 μL of 3,3′,5,5′tetramethylbenzidine (TMB) plus hydrogen peroxide. The reaction was stopped after 30 min of incubation by the addition of 50 μL of 1 N HCL. Sample absorbance at 450 nm was compared to absorbance of a known set of standards.

2.4. Fast/fed response

The effect of fasting was investigated in juvenile channel catfish ($n=120$) stocked into six 76-L aquaria. Fish were stocked at a density of 20 individuals per tank. The fish were fed to satiety once daily during a two-week acclimation period, and all individuals were weighed at the start of the experimental period (week 0). Treatments (fed and fasted) were randomly assigned to the six tanks. Fish in three of the tanks were fed to satiety once daily for 4 weeks (28 days). The remaining three tanks were fasted for the entire 4-week period. At the

conclusion of week 4, three individuals from each tank were randomly selected, weighed, and euthanized in a 300-mg/L concentration of MS-222. Hypothalamus, pituitary, muscle, and liver tissue were collected and stored as described above.

The effect of fasting on hepatic growth hormone receptor (GHR) expression normalized against a stable expressed reference gene vs. a non-stable expressed reference gene was examined following the determination of reference gene stability (Vandesompele et al., 2002).

2.5. Stress response

The effect of stress was investigated in channel catfish juveniles (n=160) stocked into eight 76-L tanks. Each tank was stocked to a density of 20 fish per tank. The fish were acclimated for a 2-week period and fed to satiety once daily. Following acclimation, fish were fasted for 48 h prior to an acute low-water stressor. Tanks were rapidly drained to an approximate depth of 1.3 cm in order to induce a low-water stress response. Low-water levels were maintained for a period of 1 h, then tanks were refilled to their initial levels. Tissue samples were collected at three separate time points. Pre-stress samples were taken prior to the stressor, post-stress samples were taken 1 h following the initiation of the stressor, and recovery samples were taken 1 h following the removal of the low-water stressor. For all treatment groups, three fish from each tank were rapidly euthanized in a solution of 8 mg/L metomidate hydrochloride (Janssen Pharmaceutica, Beerse, Belgium) and 300 mg/L MS-222. Metomidate hydrochloride blocks the handling-related release of cortisol into circulation, thus decreasing plasma cortisol variability due to sampling (Small, 2003). During sampling, all fish were captured and bled within 2 min of anesthetic exposure and then hypothalamus, pituitary, and head kidney tissues were dissected. Blood samples were taken from the caudal vasculature with heparinized syringes and placed on ice until plasma separation via centrifugation. Plasma was stored at −80 °C. Plasma cortisol in channel catfish was determined by time-resolved fluoroimmunoassay which was previously validated for channel catfish (Small and Davis, 2002). Hypothalamus, pituitary, and head kidney tissues were sampled and stored as described above.

2.6. Total RNA extraction and cDNA synthesis

Total RNA was isolated from all channel catfish tissues utilized in these reference gene studies using a modified single-step RNA isolation protocol (Chomczynski and Sacchi, 1987). Briefly, a maximum of 100 mg of tissue was used per total RNA extraction. These tissues were homogenized using an automated tissue homogenizer (TissueLyser, Retsch, Inc., Newtown, PA, USA), followed by subsequent centrifugation at 12,000 *g* for 15 min. Total RNA was precipitated from the aqueous phase by adding an equal volume of isopropanol and centrifugation at 12,000 *g* for 10 min. The total RNA pellet was washed twice with 1 mL of 75% ethanol. Pellets were resuspended in nuclease-free water and the quantity and quality of each sample were analyzed using a Nanodrop™ 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). All total RNA samples were subjected to DNase digestion in order to remove any residual genomic DNA contamination. These reactions were carried out using 1 U of DNase I (Ambion, Inc., Austin, TX, USA) per 5 µg total RNA, following the manufacturer's protocol. DNase-treated total RNA samples were diluted to a final concentration of 200 ng/µL and then utilized for first-strand cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). Following first-strand synthesis, all cDNA samples were diluted to a standard concentration of 200 ng/µL.

2.7. Primer and probe design/real-time PCR

Seven commonly used reference genes were utilized in this study: 18S ribosomal RNA (18S), alpha tubulin (TUBA), beta actin (ACTB), beta-2 microglobulin (B2M), embryonic elongation factor-1 alpha (EEF1A), glyceraldehyde phosphate dehydrogenase (GAPDH), and RNA polymerase II polypeptide B (POLR2B). All primers and probes for all reference genes were based on sequences published in Genbank (http://www.ncbi.nlm.nih.gov/; Table 1). Primer sets and dual-labeled fluorescent probes for all reference genes were designed using Beacon Designer software (PREMIER Biosoft International, Palo Alto, CA, USA). Growth hormone receptor primers and probe and assay conditions were those previously reported by Small et al. (2006).

Quantitative real-time PCR assays for all selected reference genes were conducted using iQ Supermix which contains: 100 mM KCl, 40 mM Tris–HCl, pH 8.4, 0.4 mM each dNTP, 50 U/mL iTaq DNA polymerase, and 6 mM $MgCl_2$, (Bio-Rad). For each assay, 0.1 µM each of both forward and reverse primers, 50 M of dual-labeled probe, and a total of 400 ng of cDNA template were added to each reaction. PCR amplifications were performed in triplicate for all samples using an iCycler iQ™ Real Time PCR Detection System (Bio-Rad). A no template control was run on each plate. The amplification profile was 95 °C for 3 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. Amplification products were quantified by comparison of experimental Ct (threshold cycle — defined as the PCR cycle where an increase in fluorescence over background levels first occurred) levels. To determine comparability between assays, PCR efficiency was determined on serially diluted standards developed by generating PCR fragments using the primers listed in Table 1, and then cloning them into the pCR4-TOPO vector (Invitrogen Corporation, Carlsbad, CA, USA). Cloned inserts were sequenced to confirm sequence identity. Concentration of each resulting plasmid was measured spectrophotometrically as above, and serial dilutions of each plasmid were used to make the standard curves for estimation of PCR efficiency. The resulting standard curves showed linear relationships between Ct values and the logarithm of input gene copy numbers, with efficiencies of 90–110%.

2.8. Data analysis

Statistical comparisons were conducted using SAS software system version 8.00 (SAS Institute, Inc., Cary, NC, USA). Differences among mean tissue Ct values for a given reference gene were determined using an analysis of variance (ANOVA) mixed-model procedure with tissue as the fixed effect and gene expression (Ct values) as the random effect.

Table 1
Real-time PCR primers and probes for measuring mRNA expression of 18S ribosomal RNA (18S), alpha tubulin (TUBA), beta actin (ACTB), beta-2 microglobulin (B2M), embryonic elongation factor-1 alpha (EEF1A), glyceraldehyde phosphate dehydrogenase (GAPDH), and RNA polymerase II polypeptide B (POLR2B) in channel catfish tissues

Gene	Primers/probes		Accession no.	Amplicon size (bp)
18S	Sense	GAGAAACGGCTACCACATCC	AF021880	128
	Antisense	GATACGCTCATTCCGATTACAG		
	Probe	GGTAATTTGCGCGCCTGCTGCC		
TUBA	Sense	AGCCATACAATTCCATCCTGACC	CB938582	95
	Antisense	GCGGCAGATGTCGTAGATGG		
	Probe	CCACACCACACTTGAGCACTCCGAC		
ACTB	Sense	CCCATCTATGAGGGTTATGCTCTG	CV988275	98
	Antisense	GCTCGGTCAGGATCTTCATCAG		
	Probe	CATTCTGCGTCTGGACCTGGCTGGC		
B2M	Sense	AAGGGATGGAAGTTTCATCTGACC	AF016042	142
	Antisense	GGAATGAAGCCCAGGAGGTTTAC		
	Probe	AGCGTCTCCTTCACTCCCACCAGCAATG		
EEF1A	Sense	TCAGTGAAGGACATCCGTCG	CB940917	129
	Antisense	AGCGTAGCCCTGAGAGATC		
	Probe	TGGCAACTTCACAGCTCAGGTCATCATCC		
GAPDH	Sense	TCTTATGAGCACTGTCCATGCC	CV990533	101
	Antisense	TAATGTTCTGGCTGGCACCAC		
	Probe	TCGCCACAGTTTACCAGAAGGACCATCA		
POLR2B	Sense	ACCGAAGATCATCCATGCCCTAA	CK423771	101
	Antisense	CACACGTAGTACAGCCTCATTGC		
	Probe	CCACAACGAGGCGGTGTTCTTCCAGTCA		

Table 2
Mean (n=4) cycle threshold (Ct) values from real-time PCR analysis of 18S ribosomal RNA (18S), alpha tubulin (TUBA), beta actin (ACTB), beta-2 microglobulin (B2M), embryonic elongation factor-1 alpha (EEF1A), glyceraldehyde phosphate dehydrogenase (GAPDH), and RNA polymerase II polypeptide B (POLR2B) mRNA expression in multiple channel catfish tissues

Tissue	Cycle threshold (Ct)						
	18S	TUBA	ACTB	B2M	EEF1A	GAPDH	POLR2B
Brockman bodies	11.3	25.6	19.8	23.4	25.4	22.8	29.5
Gall bladder	9.8	21.4	15.8	20.8	23.5	19.5	26.3
Gill	9.9	20.9	15.4	17.8	21.8	20.9	25.6
Head kidney	9.3	21.3	14.7	19.1	21.5	31.0	25.8
Heart	9.8	21.5	16.8	20.4	15.5	14.3	26.0
Hypothalamus	10.7	20.2	16.0	21.0	22.7	26.8	25.1
Intestine	11.2	23.0	16.8	19.6	23.6	20.3	27.5
Liver	10.4	24.2	20.4	22.9	22.8	17.7	28.3
Muscle	10.7	20.4	16.6	23.7	16.0	13.6	26.9
Ovary	11.2	20.4	14.2	23.0	21.3	16.4	20.6
Pituitary	11.0	22.5	17.7	20.2	23.0	28.6	26.4
Spleen	10.2	21.6	15.1	19.0	22.0	26.5	26.0
Stomach	9.7	23.1	15.9	21.2	22.8	21.9	27.3
Testis	9.7	21.7	16.1	20.5	21.8	21.7	25.1
Trunk kidney	9.8	21.1	14.7	19.9	22.2	18.5	23.8
pSEM*	0.14	0.08	0.11	0.16	0.16	0.16	0.14
P**	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

* Pooled standard error.
** Probability of differences in gene expression between tissues within a column.

Assumptions for homogeneity of variance and normality of the data were tested by examination of correlation between absolute residuals and predicted values, and the Shapiro–Wilk test for normality. Data not meeting the assumptions for homogeneity of variance or normality were log transformed prior to ANOVA. When significant differences were found using ANOVA, pair-wise contrasts using Fisher's LSD test identified differences at the 5% significance level. Tissue specific differences at the 5% level between physiological states (i.e. LHRH-injected vs. control; fed vs. fasted; pre-stress vs. post-stress) were determined by a comparison of the means using a Student's t-test.

The applet geNorm was used to calculate gene expression stability (M), which is the mean pair-wise variation for a gene from other tested genes (Vandesompele et al., 2002). Using this applet, ranking of tested genes was accomplished by stepwise exclusion of genes with the highest M values, such that a higher M value indicated greater variation in gene expression.

3. Results

3.1. Tissue expression

Cycle threshold values differed significantly ($P<0.0001$) among different tissues for each reference gene analyzed (Table 2). Of the seven reference genes evaluated, 18S appeared to consistently have higher transcript levels among tissues, as indicated by lower Ct values. POLR2B appeared to have the highest Ct values, and TUBA was the least variable based on the pooled standard error of the mean. Calculation of gene expression stabilities (M) across all 15 channel catfish tissues resulted in M values exceeding 2.0, several fold higher than the many M values calculated within individual tissues (Table 3). A high M value is characteristic of an unstable or differentially expressed gene (Vandesompele et al., 2002). Within individual tissues, expression stability values were much lower ranging from a low of 0.104 for ACTB and GAPDH in the ovary to 1.706 for GAPDH in the spleen. Comparison of reference gene expression stability ranking yielded no obvious pattern of preference as a stable reference gene.

3.2. Effects of exogenous LHRH treatment

Circulating plasma estradiol levels increased significantly ($P<0.05$) in female channel catfish receiving a single exogenous LHRH injection at 100 µg/kg of body mass. Average estradiol concentration was 26.6 ± 9.2 ng/mL for the LHRH-treated group and 2.1 ± 0.1 ng/mL for the control group (PBS injection only). Transcription of the seven reference genes was measured in pituitary, hypothalamus, liver, and ovarian tissues (Table 4). Levels of 18S, TUBA, ACTB, EEF1A, and GAPDH transcription in these tissues did not differ significantly between the control (PBS-injected) and LHRH-injected catfish. Levels

Table 3
Mean expression stability M (calculated using the geNorm applet; Vandesompele et al., 2002) for seven reference genes among all and within 15 channel catfish tissues (n=4)

	18S	TUBA	ACTB	B2M	EEF1A	GAPDH	POL2B
All tissues	2.278 (2)	2.091 (1)	2.418 (4)	2.689 (5)	2.974 (6)	5.179 (7)	2.391 (3)
Brockman bodies	0.754 (3)	0.905 (5)	0.659 (1)	0.769 (4)	0.948 (6)	1.307 (7)	0.694 (2)
Gall bladder	0.616 (5)	0.460 (4)	0.384 (2)	0.390 (3)	0.390 (3)	0.667 (6)	0.351 (1)
Gill	0.497 (1)	0.701 (5)	0.580 (4)	0.575 (3)	0.814 (7)	0.787 (6)	0.518 (2)
Head kidney	0.561 (1)	0.808 (6)	0.573 (3)	0.603 (4)	0.680 (5)	1.545 (7)	0.562 (2)
Heart	0.454 (6)	0.568 (7)	0.371 (3)	0.401 (4)	0.416 (5)	0.309 (1)	0.356 (2)
Hypothalamus	1.012 (7)	0.420 (2)	0.480 (5)	0.412 (1)	0.711 (6)	0.446 (3)	0.468 (4)
Intestine	0.909 (6)	0.643 (4)	0.619 (3)	0.852 (5)	0.563 (2)	1.421 (7)	0.558 (1)
Liver	0.658 (6)	0.512 (1)	0.565 (3)	0.571 (4)	0.975 (7)	0.638 (5)	0.562 (2)
Muscle	0.513 (2)	0.797 (5)	0.950 (7)	0.823 (6)	0.674 (4)	0.637 (3)	0.504 (1)
Ovary	0.282 (3)	0.350 (4)	0.104 (1)	0.402 (5)	0.537 (6)	0.104 (1)	0.174 (2)
Pituitary	0.837 (7)	0.475 (2)	0.806 (6)	0.426 (1)	0.564 (5)	0.540 (4)	0.487 (3)
Spleen	0.582 (1)	1.086 (6)	0.668 (3)	0.684 (5)	0.677 (4)	1.706 (7)	0.649 (2)
Stomach	1.089 (5)	0.922 (3)	1.076 (4)	0.862 (1)	1.111 (6)	2.770 (7)	0.888 (2)
Testes	0.737 (7)	0.504 (2)	0.530 (3)	0.618 (5)	0.691 (6)	0.574 (4)	0.475 (1)
Trunk kidney	0.897 (5)	0.688 (2)	0.767 (3)	0.669 (1)	0.862 (4)	0.980 (6)	1.389 (7)

Numerical ranking of expression stability from most to least stable within a row is provided in parentheses. A higher M value indicates greater variation in gene expression.

Table 4
Mean (±SEM; n=3) mRNA transcription levels (Ct values) of reference genes (18S ribosomal RNA (18S), alpha tubulin (TUBA), beta actin (ACTB), beta-2 microglobulin (B2M), embryonic elongation factor-1 alpha (EEF1A), glyceraldehyde phosphate dehydrogenase (GAPDH), and RNA polymerase II polypeptide B (POLR2B)) in reproductively significant tissues of channel catfish 12-h post-injection with PBS (control) or LHRH (100 μg/kg BW)

Tissue	Treatment	Reference gene mRNA transcription levels (Ct value)						
		18S	TUBA	ACTB	B2M	EEF1A	GAPDH	POLR2B
Pituitary	Control	8.1±0.03	20.8±0.09	17.8±0.20	19.0±0.08	20.8±0.13	27.5±0.16	25.9±0.09
	LHRH	8.5±0.18	20.3±0.08	17.4±0.20	19.3±0.11	21.1±0.28	27.4±0.53	26.0±0.10
		P=0.57	P=0.45	P=0.22	P=0.12	P=0.34	P=0.91	P=0.45
Hypothalamus	Control	9.1±0.37	19.4±0.10	16.6±0.12	19.0±0.04	21.4±0.16	27.1±0.16	25.6±0.18
	LHRH	9.1±0.13	19.4±0.10	16.5±0.06	18.6±0.05	21.2±0.19	26.9±0.10	25.4±0.06
		P=0.80	P=0.95	P=0.63	P=0.0026*	P=0.42	P=0.29	P=0.38
Liver	Control	8.5±0.28	24.1±0.19	20.4± 0.21	20.8±0.26	20.8±0.32	17.8±0.27	27.8±0.18
	LHRH	8.6±0.06	23.9±0.29	20.8±0.46	21.2±0.40	21.2±0.13	18.2±0.20	28.4±0.06
		P=0.96	P=0.73	P=0.44	P=0.53	P=0.34	P=0.37	P=0.028
Ovary	Control	8.6±0.32	21.4±0.28	17.0±0.58	20.0±0.37	21.8±0.14	19.3±0.50	24.2±0.76
	LHRH	8.9±0.23	20.7± 0.43	16.8±0.35	19.6±0.26	21.6±0.32	18.8±0.82	23.0±0.52
		P=0.089	P=0.22	P=0.76	P=0.42	P=0.53	P=0.64	P=0.28

* Differences between mean transcription levels of control and LHRH-injected fish were considered statistically significant at $P \leq 0.05$.

of B2M mRNA were significantly different between treatment groups in the hypothalamus (P=0.0026), with LHRH treatment resulting in increased hypothalamic B2M transcription, but no differences in B2M transcription were observed in pituitary, liver, and ovarian tissues. Catfish injected with LHRH also demonstrated decreased POLR2B transcription in liver (P=0.028) compared to controls, but no significant differences in POLR2B expression were observed in pituitary, hypothalamus, and ovarian tissues.

3.3. Effects of food depredation

Average mass of catfish fed daily to satiety at the conclusion of the four-week period was significantly (P<0.05) higher (89.6±13.8 g) compared to the average mass of the fasted catfish (31.5±1.6 g). Expression of all seven reference genes was observed in each of the somatotropic axis tissues examined (Table 5). There was no effect of fasting on expression of any of the seven reference genes in the pituitary and hypothalamus. However, both TUBA and POLR2B were upregulated in muscle as a result of fasting, as indicated by lower Ct values, while the other genes did not differ between treatment groups. In the liver, only 18S was unaffected by fasting and each of the remaining six reference genes had lower Ct values, and thus, higher levels of expression in fasted catfish relative to fed catfish.

3.4. Effects of low-water stressor

Indicative of stress, plasma cortisol levels significantly increased following the 1-h low-water stress event (P<0.05) but, after a 1-h recovery period, returned to concentrations that were not significantly different from those observed prior to the stress event (P<0.05). Specifically, plasma cortisol levels were 8.6±2.4 ng/mL prior to the 1-h low-water stress event (Pre-stress), 60.4±5.9 ng/mL following stressor (Post-stress), and 6.2±2.2 ng/mL following a 1-h recovery period (Recovery). Within the three tissues analyzed in the stress axis (hypothalamus, pituitary, and head kidney), stress had no immediate affect (Post-stress) on the expression of any of the seven reference genes (Table 6). After recovery, there were no significant changes in reference gene expression in the hypothalamic tissue, but TUBA, ACTB, and GAPDH mRNA levels were significantly higher than pre-stress levels in pituitary tissue whereas TUBA and POLR2B were significantly lower than pre-stress levels in head kidney tissue. There was no significant variation in 18S, B2M, or EEF1A gene expression in any tissues.

3.5. Reference gene dependent interpretation of GHR mRNA response to fasting

A significant effect of reference gene stability on interpretation of data was demonstrated when normalizing hepatic GHR mRNA expression in fed and fasted fish to either 18S or GAPDH (Fig. 1). As shown in Table 5, hepatic 18S expression was unaffected by fasting relative to fed fish. On the contrary, hepatic GAPDH expression was affected by fasting to the greatest extent of the reference genes examined. When normalization of GHR copy number/18S copy number was performed, a significant decrease in hepatic GHR expression was observed (Fig. 1). Normalization of GHR expression

Table 5
Mean (±SEM; n=3) mRNA transcription levels (Ct values) of reference genes (18S ribosomal RNA (18S), alpha tubulin (TUBA), beta actin (ACTB), beta-2 microglobulin (B2M), embryonic elongation factor-1 alpha (EEF1A), glyceraldehyde phosphate dehydrogenase (GAPDH), and RNA polymerase II polypeptide B (POLR2B)) in tissues of the somatotropic axis of channel catfish fed or fasted for 4 weeks

Tissue	Treatment	Reference gene mRNA transcription levels (Ct value)						
		18S	TUBA	ACTB	B2M	EEF1A	GAPDH	POLR2B
Pituitary	Fed	8.7±0.10	21.6±0.09	17.5±0.14	20.2±0.10	22.3±0.19	27.3±0.67	26.2±0.27
	Fasted	8.9±0.28	21.6±0.04	17.3±0.06	20.5±0.26	22.1±0.06	27.4±0.08	26.3±0.11
		P=0.49	P=0.94	P=0.27	P=0.36	P=0.47	P=0.81	P=0.72
Hypothalamus	Fed	8.7±0.12	19.4±0.12	16.2±0.21	21.7±0.29	22.7±0.11	26.8±0.48	25.5±0.22
	Fasted	8.6±0.24	19.5±0.04	16.1±0.15	21.4±0.17	22.6±0.32	27.2±0.08	25.2±0.19
		P=0.78	P=0.43	P=0.95	P=0.33	P=0.76	P=0.47	P=0.34
Liver	Fed	9.5±0.15	23.0± 0.24	19.6±0.30	23.3±0.41	21.9±0.34	18.0±0.08	27.7±0.18
	Fasted	9.2±0.09	21.4±0.01	18.5±0.06	21.9±0.20	20.5±0.07	15.9±0.07	26.8±0.08
		P=0.11	P=0.003*	P=0.02*	P=0.04*	P=0.01*	P= 0.00004*	P=0.009*
Muscle	Fed	9.7±0.48	21.1±0.27	17.8±0.66	26.0±0.07	16.5±0.29	15.3±0.24	28.1±0.16
	Fasted	10.6±0.26	19.8±0.04	18.6±0.18	25.0±0.52	16.0±0.08	15.4±0.15	27.2±0.11
		P=0.18	P=0.008*	P=0.26	P=0.12	P=0.18	P=0.91	P=0.04*

* Differences between mean transcription levels of fed and fasted fish were considered statistically significant at $P \leq 0.05$.

Table 6
Mean (±SEM; n=3) mRNA transcription levels (Ct values) of reference genes (18S ribosomal RNA (18S), alpha tubulin (TUBA), beta actin (ACTB), beta-2 microglobulin (B2M), embryonic elongation factor-1 alpha (EEF1A), glyceraldehyde phosphate dehydrodehase (GAPDH), and RNA polymerase II polypeptide B (POLR2B)) in tissues of the hypothalamic–pituitary–interrenal (HPI) axis of channel catfish pre-stress (Pre-), following 1 h of stress (Post-), and following 1 h of recovery (Recovery)

Tissue	Treatment	Reference gene mRNA transcription levels (Ct value)						
		18S	TUBA	ACTB	B2M	EEF1A	GAPDH	POLR2B
Pituitary	Pre-	7.7±0.12	22.7±0.10	17.6±0.18	19.7±0.20	23.1±0.20	31.6±0.07	27.4±0.07
	Post-	8.1±0.65	22.6±0.33	17.4±0.53	20.0±0.26	23.4±0.26	31.4±0.40	27.4±0.04
		P=0.51	P=0.67	P=0.78	P=0.55	P=0.44	P=0.67	P=0.77
	Recovery	7.5±0.19	21.9±0.07	16.6±0.30	19.7±0.11	22.8±0.14	30.9±0.09	27.4±0.02
		P=0.56	P=0.003*	P=0.05*	P=0.91	P=0.28	P=0.005*	P=0.85
Hypothalamus	Pre-	7.5±0.19	20.3±0.08	16.1±0.16	20.8±0.18	22.9±0.19	29.5±0.95	26.5±0.10
	Post-	7.7±0.29	20.5±0.01	16.0±0.17	20.7±0.08	23.1±0.15	30.5±0.08	26.2±0.10
		P=0.43	P=0.21	P=0.61	P=0.67	P=0.52	P=0.35	P=0.17
	Recovery	8.0±0.17	20.4±0.07	15.9±0.21	20.8±0.09	22.8±0.04	30.6±0.16	26.6±0.06
		P=0.99	P=0.86	P=0.51	P=0.99	P=0.84	P=0.32	P=0.32
Head kidney	Pre-	7.6±0.10	21.5±0.08	15.4±0.28	19.1±0.25	21.5±0.33	33.5±1.39	26.9±0.15
	Post-	7.7±0.13	21.7±0.15	15.6±0.03	19.1±0.18	21.9±0.34	33.2±2.08	27.0±0.14
		P=0.61	P=0.28	P=0.37	P=0.99	P=0.86	P=0.92	P=0.90
	Recovery	8.0±0.22	21.8±0.06	16.0±0.21	19.4±0.28	22.5±0.12	35.2±0.61	27.6±0.10
		P=0.18	P=0.03*	P=0.16	P=0.57	P=0.17	P=0.32	P=0.02*

* Differences between Pre-stress mean transcription levels and those of Post- and Recovery levels, respectively, were considered statistically significant at $P \leq 0.05$.

to GAPDH, however, suggested no effect of fasting on GHR expression.

4. Discussion

This investigation into reference gene stability in channel catfish demonstrated that among seven genes commonly used for normalization of real-time PCR results, none was consistently expressed among various tissue types. Further examination showed that the physiological state of an animal could also affect reference gene expression in a tissue specific manner. Our results supported prior research in human subjects that showed that the choice of reference gene was highly specific to the experimental model (Dheda et al., 2004). The human research demonstrated experimentally-induced directional shifts in 12 out of 13 reference genes, including ACTB, GAPDH, B2M, and EEF1A, that prevented reliable normalization. In Atlantic halibut (*Hippoglossus hippoglossus*), differential shifts in the expression of several common reference genes were observed during embryonic and larval development (Fernandes et al., 2008).

The relatively high M values for all reference genes compared among different catfish tissue types in the current study present a compelling argument against using any of these reference genes to normalize target gene expression across tissues. An alternative is normalization to total RNA, a controversial approach which has been supported and advocated for use both *in vitro* and *in vivo* (Bustin, 2000). Normalization to total RNA in itself does not control for errors introduced by differences in enzymatic efficiency or RNA quality, and accurate and reliable methods of RNA quantification are essential. With proper sample handling and RNA quantification, normalization to total RNA could be an acceptable alternative for reporting target gene expression when comparing among a variety of tissues in channel catfish.

Within tissue types, our data support the cautious use of reference genes for normalization. Several mathematical models, such as REST (Pfaffl 2001), GeNorm (Vandesompele et al., 2002) and Normfinder (Andersen et al., 2004), have been developed to validate the statistical validity of reference gene selection. Using the GeNorm applet, Vandesompele et al. (2002) present an argument for using a minimum of two and preferably three reference genes for normalization. Although more than one reference gene may appear ideal, it is not often feasible. Gene validation can be time consuming, costly and may not be feasible when samples and budgets are limited. Our study takes advantage of the GeNorm application to estimate and rank reference gene stability (M) in channel catfish tissues and utilizes the stability values together with statistical tests to evaluate expression instability in catfish tissues resulting from experimental manipulation. These results facilitate the use of one or more reference genes for normalization in similar studies with channel catfish, but do not suggest that further validation is unnecessary. On the contrary, these data clearly

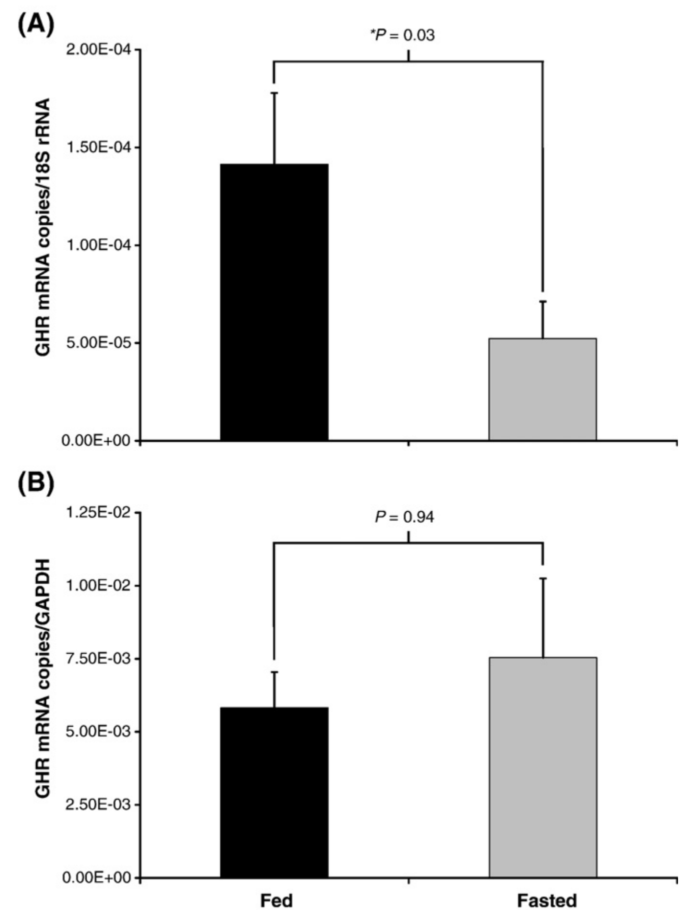

Fig. 1. The effect of reference gene choice on experimental results is demonstrated by normalizing hepatic growth hormone receptor (GHR) mRNA expression in fed and fasted fish to either (A) 18S, a reference gene stably expressed during fasting, or (B) GAPDH, a reference gene differentially expressed during fasting. Normalization of GHR to 18S indicates a significant (P<0.05; n=3) decrease in hepatic GHR expression, while normalization to GAPDH incorrectly suggests no effect of fasting on GHR expression.

demonstrate the need for validation of reference gene choice by demonstrating tissue and experiment specific alterations in reference gene stability.

The selection of valid reference genes for data normalization can be a difficult and costly task, and none of the proposed solutions appears ideal, especially across a plethora of different experimental conditions. Few studies of reference gene stability currently exist for fish. Jorgensen et al. (2006) reported a search of PubMed for real-time PCR studies with fish and showed that among 65 publications, ACTB was used for normalization in over 50% of the studies, and 18S was used in about 30% of studies. They also reported greater than 95% of the studies examined used only one reference gene for normalization. The selection of these two genes undoubtedly results from their high prevalence in non-fish literature (Bustin, 2002; Li et al., 2005; Penning et al., 2007; Dheda et al., 2004; Nygard et al., 2007).

ACTB mRNA is ubiquitously expressed in most animal cell types and has long been used as an internal standard for RNA quantification. As such, there is experimental support for its use as a quantitative reference for RT-PCR (Kreuzer et al., 1999). This support is contradictory, however, to evidence of directional shifts in expression under differing experimental conditions and tissues, including in human breast epithelial cells (Spanakis 1993); canine myocardium (Carlyle et al., 1996); and various porcine tissues (Foss et al., 1998). More recently ACTB has been both advocated (i.e. developmental expression studies in Atlantic halibut; Fernandes et al., 2008) and rejected (i.e. C-28/12 chondrocytes; Toegel et al., 2007) as a quantitative reference for real-time PCR. The apparent paradox in the suitability of ACTB as a reference gene is also reflected in the variation in catfish. ACTB mRNA expression is dependant upon induced physiological state and tissue type. Under fasting conditions ACTB was not a useful quantitative reference when examining hepatic gene expression or pituitary gene expression following acute stress. In light of these results, the overwhelming number of fish experiments using ACTB as a quantitative reference for examining hepatic gene expression under altered diet or feeding regimes should be revisited since validation of normalization procedures has typically not been indicated. Our example using differentially expressed GAPDH mRNA expression to normalize hepatic GHR expression demonstrates how selection of the wrong reference gene can significantly impact the interpretation of the data and yield contrary results.

The second most common reference gene for real-time PCR data normalization in the fish literature is 18S (Jorgensen et al., 2006). Ribosomal RNAs make up between 85 and 90% of total cellular RNA. Because their expression has been shown to be fairly stable under conditions affecting mRNA expression (Barbu and Dautry 1989) they have been widely used as quantitative references for gene expression. Earlier studies with rat (de Leeuw et al., 1993), human (Mansur et al., 1993), and mouse (Bhatia et al., 1994) tissues and cells found rRNAs to be superior to common housekeeping genes (e.g. ACTB and GAPDH) for normalizing mRNA expression. However, ribosomal RNAs are not without fault. Ribosomal RNA transcription can also be affected by experimental conditions such as certain biological factors and drugs (Spanakis 1993). In channel catfish, 18S was not differentially expressed under any of the conditions evaluated, although it was the least stable of the seven genes tested in both the hypothalamus and ovary. One drawback of using 18S as a quantitative reference is that the transcript contains no introns, thus a real-time PCR assay can be confounded by genomic DNA contamination. Another drawback is the much greater level of expression relative to target mRNAs. In the present study, mean Ct values for 18S rRNA, typically 8–11, were sometimes less than 40% of the evaluated reference genes. In channel catfish, target gene expression is typically much lower, yielding Ct values in the 20–35 range (Ct data unpublished). Because of the great difference in expression between rRNA and most target genes, normalization to rRNA may not be sufficient to detect minute, physiologically-relevant differences in gene expression.

GAPDH, while less frequently utilized in fish research, has been prevalent in the literature of non-fish species as a reference gene for normalization of real-time PCR data, despite publications demonstrating its unsuitability as a reference gene (Ke et al., 2000, Suzuki et al., 2000; Zhu et al., 2001). The poor stability of GAPDH mRNA during experimental manipulation is suggested to result from its function in nuclear RNA export, DNA replication, DNA repair, exocytotic membrane fusion, cytoskeletal organization and phosphotransferase activity (Bustin 2002). Thus, GAPDH would likely be sensitive to many perturbations in cellular homeostasis. Despite the apparent controversy regarding GAPDH as a quantitative reference for real-time PCR in other species it was found to be acceptable under certain conditions and in specific tissue types of channel catfish. Stability (M) values for GAPDH in the heart and ovary were low; however like ACTB, GAPDH mRNA was differentially expressed in the liver of fasted catfish and the pituitary of catfish following acute stress.

The remaining four reference genes (TUBA, B2M, EEF1A, and POL2B) evaluated in the current study have been used to a lesser extent as quantitative references for real-time PCR in published studies with fish. They have, however, been advocated for use as quantitative references for the normalization of real-time PCR data. In horses, TUBA was found to be highly stable in normal skin, while B2M was most stable in sarcoids (Bogaert et al., 2006). The B2M gene also ranked high in expression stability for human neuroblastoma, fibroblast, bone marrow, and normal pooled tissues (Vandesompele et al., 2002). In Atlantic salmon, EEF1A stability (M) ranked highest, together with 18S, in both normal and virus-infected tissues (Jorgensen et al., 2006). EEF1A was also one of the most stably expressed genes observed during developmental studies of larval flatfish (Infante et al., 2008). The POL2B gene was advocated by Radonić et al. (2004) for use in normalizing data from different human tissues and CCRF-HSB-2 cells. These experiments showed that "classical" normalizers (e.g. ACTB, GAPDH, and rRNAs) were unsuitable because their transcription was differently regulated in various experimental settings and different tissues (Radonić et al., 2004). Of these four genes, POL2B consistently ranked among the most stable in the various channel catfish tissues; however, differential expression was observed in various tissues following experimental manipulation (i.e. LHRH injection, fasting, and acute stress).

Although there is no clear reference gene "winner" for use in all physiological studies of channel catfish, much can be garnered from the present research regarding reference gene selection; and not only for channel catfish. Like many other studies, including some discussed here, our results suggest ribosomal RNA (i.e. 18S) to be a relatively stable quantitative reference for real-time PCR data, with some exceptions. With regard to experimentally induced differential expression, no significant changes in expression were observed in 18S relative to control samples for any of the experimental situations evaluated (i.e. LHRH injection, fasting, and acute stress). These data would seem to suggest that 18S is the best choice for normalizing real-time PCR data collected from channel catfish tissues during experimentation. However, Ct values for 18S ribosomal RNA were 2–3 times less than most genes of interest for physiological studies, and subtraction of the baseline value potentially obscures minute variations in the gene of interest. Thus, 18S should be used when no other appropriate quantitative reference can be identified.

For channel catfish, our results demonstrate the importance of validating normalization procedures for real-time PCR, and this is supported by the literature for other species. The dearth of published comprehensive protocols for normalization and respective reference gene validation creates a serious problem for the interpretation of real-time PCR results. Without proper validation of the normalization procedure, data can be misinterpreted, as our results demonstrate for hepatic GHR expression in fasted catfish. While real-time PCR is an extremely delicate and sensitive technique with numerous variables to be controlled, it is also a relatively easy assay to perform. Because of the nature of this assay, it cannot be overstated that selection of stably

and non-differentially expressed reference genes for normalization of real-time PCR data is extremely important if the results are to be reproducible and conclusions supportive of the data.

5. Conclusion

This manuscript is the first to describe the evaluation of multiple reference genes for normalizing real-time PCR data generated from *in vivo* studies of channel catfish. It is also the first, to our knowledge, to conduct such evaluations for any fish species under experimental conditions affecting the commonly studied reproductive, growth, and stress axes. Although none of the evaluated reference genes was suited for all tissue comparisons or all experimental conditions, one or more was found to be acceptable in each situation examined, excepting a comparison across multiple tissue types.

The results of this study emphasize the absolute requirement for validation of real-time PCR data normalization procedures and the importance of adequately describing those procedures and respective results when interpreting and publishing the physiological relevance of real-time PCR data. Our example using GAPDH to normalize hepatic GHR expression makes evident the significant impact normalizing to a differentially expressed gene has on interpretation of the data. Basic knowledge of stabilities and expression of select reference genes across a variety of channel catfish tissues are provided in this manuscript, thus serving as a guideline for reference gene selection. There are many strategies for real-time PCR data normalization not discussed in this manuscript, but regardless of the strategy used, the selection of reference genes must be properly validated for particular tissue or cell types and particular experimental models for proper interpretation and repeatability.

Acknowledgements

The authors wish to express their thanks to Mrs. Monica Wood and Mr. Jimmie Warren for their technical assistance in the conduct of this experiment. Mention of trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply approval to the exclusion of other products that may be suitable.

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