

Intercalibration of four spectrofluorometric protocols for measuring RNA/DNA ratios in larval and juvenile fish

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Abstract

The ratio of tissue RNA to DNA (R/D) is a widely used index of recent growth and nutritional condition in larval and juvenile fish. To date, however, no standard technique for measuring nucleic acids has been adopted. Because methodological details can affect the estimate of R/D, researchers using different analytical protocols have been unable to compare ratios directly. Here, we report on the results of an international interlaboratory calibration of 4 spectrofluorometric protocols to quantify nucleic acids. Replicate sets of 5 tissue samples and 2 standards (common standards) were supplied to each of 5 researchers for analysis with their own methods and standards. Two approaches were evaluated for mitigating the observed differences in values: 1) the use of common nucleic acid standards and 2) standardizing to a common slope ratio (slope of DNA standard curve/slope of RNA standard curve or $m_{\text{DNA}}/m_{\text{RNA}}$). Adopting common standards slightly reduced the variability among protocols but did not overcome the problem. When tissue R/Ds were standardized based on a common $m_{\text{DNA}}/m_{\text{RNA}}$ slope ratio, the variance attributed to analytical protocol decreased dramatically from 57.1% to 3.4%. We recommend that the ratio of the slopes of the standard curves be provided to facilitate intercomparability of R/D results among laboratories using different spectrofluorometric methods for the analysis of nucleic acids in fish.

Introduction

The ratio of tissue RNA to DNA (R/D) has proven to be a reliable estimator of recent growth and nutritional condition of larval and juvenile fish (e.g., review by Ferron and Leggett 1994; Folkvord et al. 1996; Rooker and Holt 1996; Clemmesen et al. 1997; Chícharo 1998; Heyer et al. 2001; Peck et al. 2003;

Buckley et al. 2004). The amount of RNA in a cell varies in proportion to protein synthesis, whereas DNA concentrations remain fairly constant, even during starvation. Thus R/D is an indicator of the protein-synthesizing potential of a cell (Young 1970; Henshaw et al. 1971; review by Bulow 1987).

As the use of the R/D index in fish has increased, so has the number of analytical protocols for measuring nucleic acids. The first published protocol was a spectrophotometric method (Schmidt and Thannhauser 1945) later modified by Munro and Fleck (1966) and adapted by Buckley (1979). More recent analytical protocols are based on the enhanced fluorescence of dyes (fluorophores) that specifically bind to nucleic acids. The main advantage of these newer methods is a substantial increase in sensitivity and sample throughput compared with the UV-spectrophotometric technique. For quantitative purposes, however, the fluorometric approaches are highly sensitive to methodological details: the estimation of nucleic acid concentrations and ratios are influenced greatly by factors such as the protein disso-

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Table 1. Description of tissues analyzed.

Species (sample abbreviation)	Life Stage	Sampling Location	Rearing or Collection Temperature, °C	Age, d	Average Length, mm	Tissue sampled	Total No. Individuals in Homogenate	Aliquot Concentration, dry wt mg mL ⁻¹
Houting <i>Coregonus oxyrhynchus</i> (Co)	Larval	Laboratory reared, Kiel, Germany	8	32	18	Whole fish	35	2.1
Herring <i>Clupea harengus</i> (Ch-j1, Ch-j2)	Juvenile	Field-caught, Kiel Canal, Germany (Baltic)	18	NA	40	Whole fish minus head, gut, tail fin	35	4.6
Herring <i>Clupea harengus</i> (Ch-l)	Larval	Field-caught, International Herring Larvae Survey, ICES Orkney/ Shetland area	13	NA	14	Whole fish	100	4.4
Cod <i>Gadus morhua</i> (Gm)	Juvenile	Mesocosm-reared, Flodevigen, Norway	13	70	40-60	Muscle filet	NA	4.2

NA, not available.

ciation step, standards, and fluorophores used (Caldarone and Buckley 1991; Clemmesen 1993; Canino and Caldarone 1995; Berdalet et al. 2005a,b). Consequently, methodological differences between fluorometric analyses have precluded researchers from directly comparing results between laboratories or using published models defining the relation between R/D and instantaneous growth rates. It has been argued that to directly compare results, either identical analytical methods and standards must be used or a rigorous intercalibration between the nucleic acid analysis methods must be conducted (e.g., Caldarone and Buckley 1991; McGurk and Kusser 1992; Clemmesen 1993; Grémare and Vétion 1994; Buckley et al. 1999; Esteves et al. 2000).

Here, we compare results from 4 spectrofluorometric protocols for quantifying R/D. The methodological approaches of the protocols are similar in that they all contain a step to lyse cells and dissociate proteins, use a nonspecific fluorescent dye (ethidium bromide [EB] or SYBR Green II [SYBR]), and include a treatment with 1 or 2 nucleases to partition total fluorescence into RNA and DNA components. There are, however, important differences among the techniques, particularly concerning the specific standards, protein-dissociation chemical, buffers, nuclease incubations, fluorophores, and fluorometers used. The main objective of this study was to determine whether it is possible to standardize results obtained from different spectrofluorometric protocols to allow for meaningful comparisons of the data, without evaluating the specific differences between the approaches. We first estimated nucleic acid concentrations and R/D ratios of the same 5 fish tissues using the 4 different protocols, and then investigated whether using common standards and/or a standardization factor based on the ratio of the standard curves (Berdalet et al. 2005b) could contribute to the direct comparison of the obtained R/D ratios.

Five research groups and 8 researchers were involved in this joint exercise. This unique opportunity also allowed us to

compile a list of analytical recommendations and precautions, which is presented at the end of this study.

Materials and Procedures

Preparation of Standards and Tissue Samples—Each laboratory was supplied with 1 set of standards and tissue samples. The supplied standards (common standards) were: 0.0125 mg mL⁻¹ λ-phage DNA (Boehringer Mannheim) prepared in a buffer containing 0.05 M Tris, 0.01 M EDTA, and 0.1 M NaCl at a pH of 8.0 (TEN buffer), and 0.02 mg mL⁻¹ 16S and 23S rRNA (Boehringer Mannheim) prepared in TEN buffer. Five different tissues were quantified. Tissue samples consisted of whole larval houting (*Coregonus oxyrhynchus*, abbreviated Co), whole larval herring (*Clupea harengus*, Ch-l), juvenile herring muscle (Ch-j1), centrifuged juvenile herring muscle (Ch-j2), and juvenile cod muscle (*Gadus morhua*, Gm) (Table 1). Tissues and whole fish were freeze-dried overnight and weighed before being rehydrated in TEN buffer. Co, Ch-l, and Gm tissues were homogenized for 25 s at room temperature with an Ultraturrax mixer and diluted with ice-cold TEN buffer. One-milliliter aliquots of the homogenates were stored in microcentrifuge vials at -70 °C. Ch-j tissue was shaken for 15 min at room temperature in suspension with varying-sized glass beads which were removed by centrifugation before dilution with ice-cold TEN buffer. One-half of the resulting homogenate was pipetted (1-mL aliquots) into microcentrifuge vials and stored at -70 °C (Ch-j1). The remaining homogenate was centrifuged at 3800g for 8 min at 4 °C, divided into aliquots, and frozen (Ch-j2). When all standards and tissue samples were prepared, 2 sets of the common standards and 1 vial of each of the 5 tissue homogenates were sent (on dry ice) to the participating laboratories where they were stored at -70 °C until the day of analysis.

Nucleic Acid Analysis—Five laboratories employing 4 different protocols for quantifying nucleic acids participated in the

Table 2. Description of analytical protocols used in each laboratory.

Laboratory Abbreviation	Fluorometer				Assay			Individual RNA Standard		Individual DNA Standard	
	Fluorophore	Type	Manufacturer	Excitation wavelength (nm)	Emission wavelength (nm)	Protein dissociator	Enzymes	Source	Product No.	Source	Product No.
EBsarco1	Ethidium bromide	Microplate	Bio-Tek FL500	530	590	N-lauroylsarcosine	RNase	18S, 28S rRNA calf liver	Sigma-Aldrich R0889	Calf thymus	Sigma-Aldrich D4764
EBsarco2	Ethidium bromide	Microplate	IDEXX FCA-VIP	545	575	N-lauroylsarcosine	RNase	18S, 28S rRNA calf liver	Sigma-Aldrich R0889	Calf thymus	Sigma-Aldrich D4764
EBSDS	Ethidium bromide	Microplate	LabSystems Fluoroskan Ascent	355	590	Sodium dodecyl sulfate (SDS)	RNase	16S, 23S rRNA	Boehringer Mannheim 206938	λ-phage	Boehringer Mannheim 745782
EBproK	Ethidium bromide	Cuvette	Turner 111	365	590	Proteinase K	RNase followed by DNase	Baker's yeast	Sigma-Aldrich R7125	Calf thymus	Sigma-Aldrich D1501
SYBR	SYBR Green II	Cuvette	Spectronic, Aminco-Bowman 2	490	520	N-lauroylsarcosine	RNase and DNase independently	18S, 28S rRNA calf liver	Sigma-Aldrich R0889	Calf thymus	Sigma-Aldrich D4764

study. Three laboratories were located in the United States and 1 each in Germany and Spain. Each laboratory team quantified the 5 tissue samples using both their own RNA and DNA standards (individual standards, Table 2) and the 2 common standards. In most instances, replicates of the tissues were analyzed. All 4 protocols were fluorometric based, 3 used EB, and 1 used SYBR as the fluorophore. All protocols employed 1 or 2 nucleases (RNase, DNase). Three different chemicals were used to lyse cells and dissociate proteins: 2 detergents (*N*-lauroylsarcosine and sodium dodecylsulfate [SDS]) and 1 enzyme (proteinase K). Two of the protocols used a microplate fluorometer, the other 2 a cuvette spectrofluorometer. In all cases fluorescence yield was converted to μg of nucleic acid per mL extract by comparison with standard curves constructed with both the individual and common standards.

The main components of the protocols are summarized in Table 2, and abbreviated descriptions are given below. Detailed instructions for each method can be found in the references provided within each section.

EBsarco1 and EBsarco2. Two laboratories used identical analytical protocols, differing only in the manufacturer of the microplate fluorometers, which resulted in minor differences in the wavelength maxima (Table 2). In both laboratories, each tissue sample was extracted in *N*-lauroylsarcosine (final concentration 1%) in Tris-EDTA (TE) buffer (pH 7.5). After diluting and centrifuging the samples, a portion of the supernatant was combined in a microplate with the fluorophore EB, and the total nucleic-acid fluorescence was recorded with a microplate fluorometer. RNase was then added to each well, and the plate was incubated with shaking for 20 min at room temperature before being read a second time. The resulting fluorescence was attributed to DNA. RNA concentrations were calculated from the difference in fluorescence between the first and second readings (Caldarone et al. 2001).

EBSDS. Each tissue sample was extracted in SDS (final concentration 0.01%) in TEN buffer. After centrifuging the samples, 2 aliquots of the supernatant were added to a microplate containing the fluorophore EB. RNase was added to the second aliquot, and the plate was incubated for 30 min at 37 °C and cooled to room temperature before the fluorescence of both aliquots was recorded with a microplate fluorometer. The fluorescence from the RNase-treated aliquot was attributed to DNA. RNA concentrations were calculated from the difference in fluorescence between the 2 aliquots (Malzahn et al. 2003; Belchier et al. 2004; Melzner et al. 2005).

EBproK. After centrifuging the tissue samples, portions of the supernatant were pipetted into borosilicate test tubes containing TE buffer (pH 8), ionic cofactors, the fluorophore EB, and proteinase K (10% wt:vol). Total fluorescence of each sample was recorded using a cuvette fluorometer. RNase was then added to each tube, and the samples were incubated for 30 min at 37 °C and cooled to room temperature before being read a second time. RNA concentrations were calculated from the difference in fluorescence between the first and second read-

Table 3. Standardization factor (SF_{P_i}) calculated by dividing each protocol m_{DNA}/m_{RNA} by the reference protocol (EBsarco1) m_{DNA}/m_{RNA}

Individual standards				
Laboratory	DNA standard curve slope (m_{DNA})	RNA standard curve slope (m_{RNA})	m_{DNA}/m_{RNA}	SF_{P_i}
EBsarco1	4656	1956	2.4	1.00
EBsarco2	227.9	105.2	2.2	0.92
EBSDS	50.9	33.4	1.5	0.64
EBproK	70.5	15.3	4.6	1.92
SYBR	7.5	2.0	3.9	1.63
Common standards				
Laboratory	DNA standard curve slope (m_{DNA})	RNA standard curve slope (m_{RNA})	m_{DNA}/m_{RNA}	SF_{P_i}
EBsarco1	4382	1743	2.5	1.00
EBsarco2	247.9	96.7	2.6	1.04
EBSDS	50.9	33.4	1.5	0.61
EBproK	62.7	30.1	2.1	0.84
SYBR	9.2	2.1	4.3	1.72

ings. After DNase was added to each tube, the samples were incubated and cooled as above and read a third time to estimate residual fluorescence. DNA concentrations were calculated from the difference in fluorescence between the second and third readings (Westerman and Holt 1988).

SYBR. An aliquot of each tissue sample was extracted in *N*-lauroylsarcosine (final concentration 0.5%) in TE buffer (pH 7.5). The samples were then diluted with Tris buffer and distributed into 3 borosilicate test tubes containing ionic cofactors and Tris buffer. DNase was added to 1 test tube, RNase to the second, and both enzymes to the third to estimate residual fluorescence. All enzyme treatments were incubated for 20 min at 37 °C, after which the tubes were cooled to room temperature. The fluorophore SYBR was then added and the fluorescence recorded using a cuvette spectrofluorometer. After subtracting the residual fluorescence from both the DNase- and RNase-treated assays, the remaining fluorescences were attributed to RNA and DNA, respectively (Berdalet et al. 2005a,b).

Calculations for Normalization Procedure—To investigate standardizing R/D among protocols, we tested the approach reported by Berdalet et al. (2005b) based on the ratio of the slopes of the standard curves. The rationale for their conversion factor was that for any particular fluorometric protocol, the slopes of the RNA and DNA standards determine the RNA and DNA concentrations of a sample. This method assumes that the standard curves are linear and the intercept (after subtraction of the reagent blank) is not significantly different from

zero. For all protocols in this study, the coefficients of determination for the standard curves were > 0.99 and the y -intercepts were near zero after subtraction of the reagent blank.

In all of the protocols, concentrations of the nucleic acids were determined using a linear standard curve of the form

$$F = mx + b \quad (1)$$

where F = fluorescence units, m = slope, x = concentration of the nucleic acid ([RNA] or [DNA]), and b = intercept.

Solving for the concentrations (x) of the 2 nucleic acids yields

$$[RNA] = (F_{RNA} - b_{RNA})/m_{RNA} \quad [DNA] = (F_{DNA} - b_{DNA})/m_{DNA} \quad (2)$$

Because the y -intercepts of the standard curves are non-significantly different from zero, the equations simplify to

$$[RNA] = F_{RNA}/m_{RNA} \quad [DNA] = F_{DNA}/m_{DNA} \quad (3)$$

The R/D ratio value is therefore proportional to the standard curve slope ratio (m_{DNA}/m_{RNA}):

$$[RNA]/[DNA] = (F_{RNA}/m_{RNA})/(F_{DNA}/m_{DNA}) = (F_{RNA}/F_{DNA}) * (m_{DNA}/m_{RNA}) \quad (4)$$

To standardize the slope ratios from the different protocols (P_i) to 1 protocol (reference protocol, P_{ref}), a standardization factor (SF_{P_i}) was calculated for each protocol by dividing the standard curve slope ratio of each protocol $[(m_{DNA}/m_{RNA})_{P_i}]$ by the slope ratio of the reference protocol $[(m_{DNA}/m_{RNA})_{P_{ref}}]$.

$$SF_{P_i} = (m_{DNA}/m_{RNA})_{P_i} / (m_{DNA}/m_{RNA})_{P_{ref}} \quad (5)$$

For this study, EBSarco1 was arbitrarily chosen as the reference protocol. Thus, the SF_{P_i} s were generated by dividing each protocol m_{DNA}/m_{RNA} by 2.4 when the individual standards were used or by 2.5 when the common standards were used (Table 3).

Each R/D data value from each P_i protocol was then divided by its respective SF to yield a standardized R/D value (sR/D)

$$sR/D_{P_i} = (R/D)_{P_i} / SF_{P_i} \quad (6)$$

This procedure is mathematically equivalent to the conversion factor described in Berdalet et al. (2005b).

Statistical Analysis—Tissue samples were analyzed in replicate ($n = 2$ to 10) by all laboratories except EBSarco2. For all comparisons among the 5 laboratories, mean values for each tissue type from each laboratory were used. For all comparisons among the 4 protocols, a mean of EBSarco1 and EBSarco2 mean values was used to allow for equal weighting of the 4 different protocols. Post-hoc inspection of the results indicated concerns (abnormally high residual fluorescence and CVs) with Co tissue samples from 1 laboratory (EBproK), indicating that results for this particular sample may be unreliable; therefore these data were not included in any analyses.

A general linear model was used to partition the variation in the study to within-protocol and between-protocol sources. Variance components for a nested treatment design (tissue sample, protocol nested in tissue sample, replicate nested in protocol) using maximum-likelihood techniques were esti-

mated. Variance components for the dependent variables (RNA concentration, DNA concentration, R/D, sR/D) were calculated with both individual and common standards using the model

$$\text{dependent variable} = \text{sample protocol}(\text{sample}) \text{ replicate}(\text{protocol})$$

All statistical analyses were carried out using SAS software version 8.02 (SAS Institute 1999).

Assessment and Discussion

Nucleic acid Concentrations—To obtain a range of nucleic acid concentrations and R/D values, we chose tissue from a variety of species and early life-history stages. When laboratories used their individual standards, RNA concentrations ranged from 21.2 $\mu\text{g mL}^{-1}$ (Ch-j2, EBSDS) to 200.8 $\mu\text{g mL}^{-1}$ (Ch-l, SYBR) (Table 4, Figure 1A), with 55.7% of the variance in RNA concentrations attributable to differences among the samples (Table 5). The remainder of the variance (44.2%) (Table 5) was due to differences among the protocols. Because the composition of a standard will determine the fluorescent quantum yield, one factor that contributed to this interprotocol variability was the different compositions of the RNAs used as standards (Table 2). Baker's yeast (Sigma-Aldrich) is a mixture of ~85% tRNA, 10 to 15% eukaryotic rRNA, and 1 to 2% mRNA, whereas 16S and 23S rRNA and 18S and 28S rRNA contain 100% highly purified prokaryotic and eukaryotic rRNA, respectively. Additionally, other differences among the protocols, such as the specific fluorophore and protein dissociation chemical used, undoubtedly contributed to the observed interprotocol differences.

When laboratories used their individual standards, 81.6% of the variance in DNA concentrations was attributable to differences among the samples (Table 5), with individual values ranging from 9.0 $\mu\text{g mL}^{-1}$ (Gm, EBproK) to 39.8 $\mu\text{g mL}^{-1}$ (Ch-l, SYBR) (Table 4, Figure 1B). Interprotocol differences accounted for 17.9% of the variance (Table 5). The individual DNA standards used by the different laboratories were similar (Table 2), which may have contributed to the lower DNA protocol variance compared to the RNA value. All of the DNA standards contained high-molecular-weight double-stranded DNA (dsDNA). Sigma-Aldrich (D4764) calf thymus DNA and Boehringer Mannheim λ -phage DNA are purified and expected to contain no low-molecular-weight or single-stranded components, and Sigma-Aldrich (D1501) calf thymus DNA consists primarily of dsDNA.

When laboratories used their individual standards, 42.5% of the variance in R/D was attributable to differences among the samples (Table 5), with individual values ranging from 0.6 (Co, EBSDS) to 11.7 (Gm, EBproK) (Table 4, Figure 2A). Interprotocol differences accounted for 57.1% of the variance (Table 5), which was a higher percentage than for either of the nucleic acid concentrations alone. A portion of this increased variance could be associated with an attribute of all ratio-based estimates: small changes in opposite directions in a numerator and denominator are amplified in a ratio.

The high variances attributed to interprotocol differences confirm that it is not meaningful to directly compare nucleic

acid concentrations or R/D values obtained using the different spectrofluorometric protocols and individual standards. The analysis also confirms that published equations incorporating R/D to calculate instantaneous growth rates cannot be directly used without an intercalibration between methods.

Two laboratories (EBsarco1, EBsarco2) used the same protocol (standards, reagents, microplates, assays, calculations), differing only in the manufacturer of the fluorometers. Paired *t* tests revealed no differences in RNA ($P = 0.18$) and DNA ($P = 0.30$) concentrations between the 2 laboratories, but a significant difference between ratio values ($P = 0.04$). Gm and Ch-l R/D values were the most different between the 2 laboratories (Table 4, Figure 2A). Some of this difference reflects the fact mentioned above: small differences in individual nucleic acid values can be amplified when a ratio is calculated. For example, the individual Gm nucleic acid concentrations were very similar between the protocols (Table 4, Figure 1A, B), but their RNA and DNA values differed slightly in opposite directions, which resulted in a ratio more different than would have been expected from examining the individual concentrations. Overall, R/D values generated by the 2 laboratories using the same protocol were comparable (Figure 2A).

It is important to note that despite the absolute differences observed in nucleic acid concentrations and R/D values among the protocols, the rank order of the tissues within each protocol was identical between laboratories (Figure 1A, B, Figure 2A). For example, each laboratory determined that Co had the lowest R/D value, followed by a group consisting of the 3 herring tissues (Ch-j2, Ch-j1, Ch-l), and ending with Gm. It is also notable that when the variance was partitioned, replicates within a protocol contributed $\leq 0.1\%$ of the variance in either nucleic acid concentration or R/D value (Table 5). The robust relative rankings, together with the excellent precision within a protocol, emphasize the effectiveness of each protocol to reliably measure relative levels of nucleic acids in fish tissues. Thus, within each laboratory, hypotheses based on relative nucleic acid concentrations and/or R/D values can be confidently tested and inferences and conclusions made.

Standardization—The consistency of the nucleic acid and R/D rankings among protocols suggested that if some appropriate standardization were developed, the different protocols could produce comparable values. We explored 2 potential options for standardizing results. First, we determined whether using common nucleic acid standards would overcome differences among protocols. Second, we tried standardizing results based on the ratio of the slopes from the 2 calibration curves.

Common standards. Being aware of the dependence of nucleic acid values on the fluorescence response of the standards, and the different formulations of nucleic acids available for use as a standard, researchers have often suggested that the use of common standards would improve intercomparability of results among laboratories (Buckley et al. 1999; Caldarone et al. 2001; Belchier et al. 2004; Melzner et al. 2005). In the pres-

Table 4. Mean RNA and DNA concentrations and R/D in each tissue for each protocol.

Sample	Protocol	n	Individual Standards							
			RNA, µg mL ⁻¹	Intralaboratory RNA CV, %	DNA, µg mL ⁻¹	Intralaboratory DNA CV, %	R/D	sR/D	Intralaboratory R/D and sR/D CV, %	
Co	EBsarco1	8	42.1	3.8	30.9	3.0	1.4	1.4	4.5	
	EBsarco2	1	35.5	NA	36.1	NA	1.0	1.1	NA	
	EBSDS	10	21.8	3.6	36.8	3.5	0.6	0.9	3.8	
	EBproK	NA	NA	NA	NA	NA	NA	NA	NA	
	SYBR	2	57.4	1.0	32.9	7.9	1.8	1.1	6.8	
	Interprotocol									
	Mean			39.4		34.4		1.2	1.1	
SD			17.8		2.1		0.6	0.1		
CV, %			45.3		6.1		49.5	13.5		
Ch-j2	EBsarco1	8	31.7	2.4	11.9	5.3	2.7	2.7	5.9	
	EBsarco2	1	30.0	NA	12.1	NA	2.5	2.7	NA	
	EBSDS	9	21.2	3.2	14.5	2.3	1.5	2.3	2.5	
	EBproK	7	49.6	3.7	10.0	3.4	4.9	2.6	5.8	
	SYBR	2	46.9	2.6	12.9	6.4	3.6	2.2	3.7	
	Interprotocol									
	Mean			37.1		12.4		3.2	2.4	
SD			13.5		1.9		1.5	0.2		
CV, %			36.2		15.1		47.1	9.2		
Ch-j1	EBsarco1	8	36.7	4.2	12.9	4.1	2.8	2.8	5.3	
	EBsarco2	1	34.7	NA	12.8	NA	2.7	3.0	NA	
	EBSDS	9	25.1	2.8	15.9	0.8	1.6	2.5	2.5	
	EBproK	7	52.5	4.1	10.1	2.6	5.2	2.7	5.6	
	SYBR	2	61.2	1.4	14.1	6.0	4.4	2.7	4.6	
	Interprotocol									
	Mean			43.6		13.3		3.5	2.7	
SD			16.3		2.4		1.6	0.2		
CV, %			37.3		18.3		46.4	6.8		
Ch-l	EBsarco1	8	115.7	1.9	32.8	2.5	3.5	3.5	1.7	
	EBsarco2	1	84.9	NA	32.1	NA	2.7	2.9	NA	
	EBSDS	10	68.3	3.3	37.6	2.4	1.8	2.8	3.2	
	EBproK	7	146.2	1.8	21.0	1.7	7.0	3.6	0.6	
	SYBR	2	200.8	0.1	39.8	2.7	5.0	3.1	2.9	
	Interprotocol									
	Mean			128.9		32.7		4.2	3.2	
SD			57.6		8.4		2.3	0.3		
CV, %			44.7		25.7		53.3	10.0		
Gm	EBsarco1	8	89.8	2.3	13.2	4.5	6.8	6.8	4.6	
	EBsarco2	1	85.7	NA	14.6	NA	5.9	6.4	NA	
	EBSDS	8	61.0	2.9	16.8	1.2	3.6	5.7	3.2	
	EBproK	7	104.7	0.9	9.0	2.5	11.7	6.0	2.4	
	SYBR	2	123.2	0.3	12.5	8.7	9.9	6.0	9.0	
	Interprotocol									
	Mean			94.1		13.1		7.9	6.1	
SD			26.4		3.3		3.6	0.4		
CV, %			28.1		25.0		45.6	6.5		
Average for all tissues										
	EBsarco1			2.9		3.9			4.4	
	EBsarco2			NA		NA			NA	
	EBSDS			3.2		2.0			3.0	
	EBproK			2.6		2.5			3.6	
	SYBR			1.1		6.3			5.4	

Continued

TABLE 4—Continued

Sample	Protocol	n	Common Standards							
			RNA, µg mL ⁻¹	Intralaboratory RNA CV, %	DNA (µg ml ⁻¹)	Intralaboratory DNA CV, %	R/D	sR/D	Intralaboratory R/D and sR/D CV, %	
Co	EBsarco1	8	41.6	4.3	31.4	3.1	1.3	1.3	5.2	
	EBsarco2	1	37.1	NA	32.5	NA	1.1	1.1	NA	
	EBSDS	10	21.8	3.6	36.8	3.5	0.6	1.0	3.8	
	EBproK	NA	NA	NA	NA	NA	NA	NA	NA	
	SYBR	2	45.1	1.9	25.4	8.1	1.8	1.0	6.4	
	Interprotocol									
	Mean		35.4		31.4		1.2	1.1		
	SD		12.1		5.7		0.6	0.1		
CV, %		34.3		18.3		49.4	11.7			
Ch-j2	EBsarco1	8	30.0	2.9	11.1	6.0	2.7	2.7	6.8	
	EBsarco2	1	30.2	NA	11.8	NA	2.6	2.5	NA	
	EBSDS	9	21.2	3.2	14.5	2.3	1.5	2.4	2.5	
	EBproK	7	23.8	3.9	10.2	3.7	2.3	2.8	6.4	
	SYBR	2	36.6	2.6	9.9	6.4	3.7	2.2	3.6	
	Interprotocol									
	Mean		27.2		11.5		2.5	2.5		
	SD		6.9		2.1		0.9	0.3		
CV, %		24.6		18.2		36.3	11.4			
Ch-j1	EBsarco1	8	35.6	4.9	12.2	4.6	2.9	2.9	5.9	
	EBsarco2	1	36.2	NA	11.7	NA	3.1	3.0	NA	
	EBSDS	9	25.1	2.8	15.9	0.8	1.6	2.6	2.5	
	EBproK	7	25.3	4.3	10.3	2.8	2.5	3.0	6.2	
	SYBR	2	47.8	1.4	10.9	6.0	4.4	2.6	4.7	
	Interprotocol									
	Mean		33.5		12.3		2.9	2.8		
	SD		10.8		2.5		1.2	0.2		
CV, %		32.2		20.6		41.3	8.1			
Ch-l	EBsarco1	8	124.2	2.0	33.3	2.6	3.7	3.7	1.8	
	EBsarco2	1	91.1	NA	30.3	NA	3.0	2.9	NA	
	EBSDS	10	68.3	3.3	37.6	2.4	1.8	3.0	3.2	
	EBproK	7	72.9	1.8	22.5	1.8	3.2	3.9	0.6	
	SYBR	2	156.8	0.1	30.8	2.7	5.1	3.0	2.8	
	Interprotocol									
	Mean		101.4		30.7		3.4	3.3		
	SD		40.9		6.2		1.3	0.4		
CV, %		40.3		20.3		39.8	13.1			
Gm	EBsarco1	8	95.1	2.5	12.5	5.0	7.6	7.6	5.1	
	EBsarco2	1	89.9	NA	13.6	NA	6.6	6.5	NA	
	EBSDS	8	61.0	2.9	16.8	1.2	3.6	6.0	3.2	
	EBproK	7	51.8	0.9	9.0	2.8	5.8	7.0	2.7	
	SYBR	2	96.2	0.3	9.7	8.7	10.0	5.8	8.9	
	Interprotocol									
	Mean		75.4		12.1		6.6	6.4		
	SD		22.3		3.6		2.7	0.6		
CV, %		29.6		29.6		40.1	9.9			
Average for all tissues										
	EBsarco1			3.3		4.2			5.0	
	EBsarco2			NA		NA			NA	
	EBSDS			3.2		2.0			3.0	
	EBproK			2.7		2.8			4.0	
	SYBR			1.3		6.4			5.3	

Concentrations were calculated with both individual and common standards. For interprotocol CVs, the mean of EBsarco1 and EBsarco2 were used as 1 protocol mean.

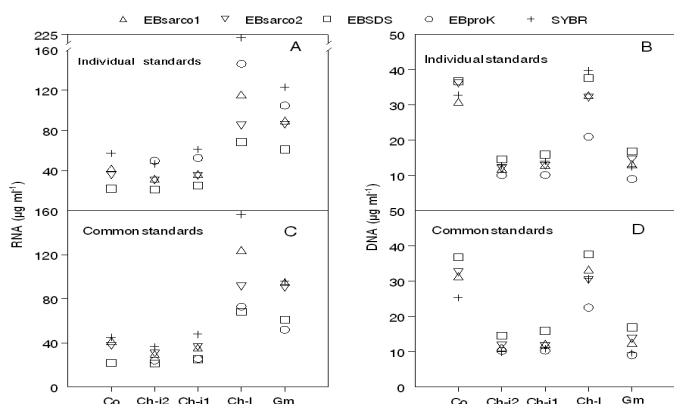


Fig. 1. Mean RNA (A, C) and DNA (B, D) concentrations ($\mu\text{g mL}^{-1}$) for each tissue analyzed by each laboratory. Concentrations were calculated using both individual (A, B) and common (C, D) standards. Abbreviations as in Tables 1 and 2.

ent study, the use of a common standard slightly decreased the interprotocol variance in RNA concentration (44.2% vs. 41.8%) (Table 5). The Baker’s yeast standard (mixture of eukaryotic RNAs, not highly purified) was the most different from the common standard source (prokaryotic rRNA, highly purified), and individual values calculated with that standard did show the greatest change with the use of a common standard (Table 4, Figure 1A, C, EBproK results). This result highlights the fact that different classes of RNA have different fluorescence responses.

The interprotocol variance in DNA concentration did not change when a common standard was used (17.9%) (Table 5, Figure 1B, D), most likely because the individual standards were very similar in composition to the common standard. It is unclear why, even when common standards were used, interprotocol variability was greater in RNA values compared with DNA values. Three of the protocols (EBsarco, EBSDS, EBproK) measure RNA through a sequential subtraction step (total fluorescence minus DNA fluorescence): any problems measuring 1 nucleic acid would have resulted in high variability in both measurements, and this was not the case. It appears that other aspects of the protocols also influence

nucleic acid concentrations. Certainly differences in the binding of the specific fluorophores to the nucleic acids, the protein dissociation chemical and concentration, enzyme incubation procedures, and handling procedures used to minimize degradation could contribute to the observed interprotocol differences. Cleaning and sterilizing procedures for glassware, plasticware, and solutions varied between laboratories but did not appear to be correlated to the amount of RNA determined.

The rank order of the nucleic acid concentrations and R/D ratios of the tissues within a laboratory was not altered by the use of common standards (Figure 1C, D, Figure 2C), and, for all 3 variables, the variance due to replicates within a protocol remained low ($\leq 0.1\%$) (Table 5). Adopting common standards did slightly reduce the interprotocol variance in R/D (57.1% vs. 47.6%) (Table 5); however, it was not enough to overcome the interprotocol comparison problem (Table 4, Figure 2C). These results indicate that in fluorometric-based procedures, possibly even small differences in a protocol can contribute to differences in R/D values. For example, despite the many similarities between the EBsarco1 and EBSDS protocols, the Gm R/D values calculated using common standards were not comparable (7.6 vs. 3.6, respectively). These findings confirm that meta-analyses of R/D data from studies using common standards but different fluorometric protocols are likely to be biased and inaccurate; this would include direct comparison of R/D values as well as interpretation of R/D-based growth rates. These precautions are not limited to the protocols tested here but would apply to any fluorometric-based nucleic acid methodology.

Common DNA/RNA slope ratio. Our second approach to standardization was based on the slopes of the DNA and RNA standard curves, as described by Berdalet et al. (2005b). The DNA/RNA slope ratio from each protocol was normalized by dividing by the EBsarco1 slope ratio (Table 3). In this study, EBsarco1 was arbitrarily chosen as the reference; in most instances, researchers would use their own slope ratio to normalize against. The resulting standardization factor (SF_{p}) was used to standardize the R/D values. This normalization procedure dramatically reduced differences in R/D values among protocols while maintaining the intersample rank order of the

Table 5. Maximum likelihood estimates of variance components of a general linear model with either RNA, DNA, R/D, or sR/D as the dependent variable, expressed as a percent of the total variance.

Independent variables	Variance components, %							
	Individual Standards				Common Standards			
	RNA	DNA	R/D	sR/D	RNA	DNA	R/D	sR/D
Samples	55.7	81.6	42.5	95.9	58.0	81.6	51.9	92.7
Protocols within a sample (interprotocol differences)	44.2	17.9	57.1	3.4	41.8	17.9	47.6	6.5
Replicates within a protocol	< 0.1	0.1	0.1	0.1	< 0.1	0.1	0.1	0.1
Error	0.1	0.3	0.3	0.7	0.2	0.4	0.5	0.7

Nucleic acid concentrations were calculated with both individual and common standards. Data were from EBsarco1, EBSDS, EBproK, and SYBR laboratories.

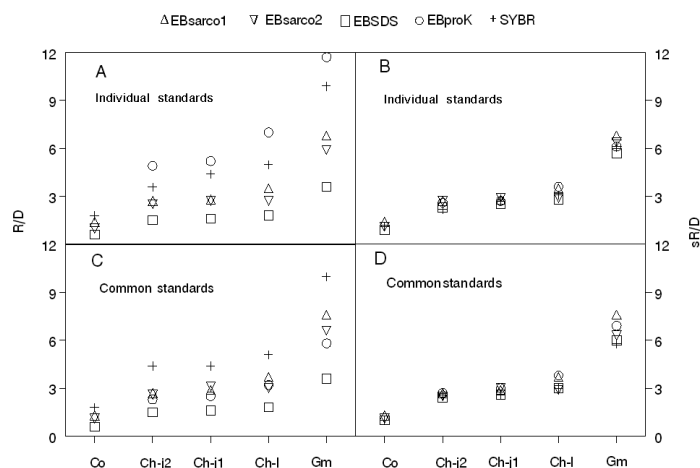


Fig. 2. Mean R/D (A, C) and sR/D (B, D) for each tissue analyzed by each laboratory. Concentrations were calculated using both individual (A, B) and common (C, D) standards. Abbreviations as in Tables 1 and 2.

tissues (Table 4, Figure 2B, D). The interprotocol variance was significantly reduced from 57.1% to 3.4% when individual standards were used, and from 47.6% to 6.5% when common standards were used (Table 5). This normalization procedure does not require the adoption of common standards or a common analytical technique, yet offers a simple convenient pathway for comparing R/D values without conducting a rigorous intercalibration between spectrofluorometric methods. For example, if lab A (with an average DNA/RNA slope ratio of 2) wants to compare their fluorometrically derived R/D results to lab B (which reported an average DNA/RNA slope ratio of 6), the results of lab B could be standardized to the results of lab A by dividing each lab B R/D by 3. The resulting standardized R/D values (sR/D) from lab B could then be directly compared to the R/D results of lab A.

Comments and Recommendations

Study Summary—In this study we have shown that standardizing tissue R/D values based on a common slope ratio procedure was effective when both common and different nucleic acid standards were used, and when nucleic acids were analyzed with different fluorometric protocols. We propose that such a standardization approach would enable investigators to more meaningfully compare R/D values among laboratories, and enable the use of published models defining the relation between R/D and instantaneous growth rates in fish. For this approach to be successful, we recommend that the average calibration curves of the standards, and the source of the standards used, be reported in future publications to facilitate interconversion of R/D values.

It is also important to mention that standardizing R/D values does not eliminate all factors which may affect interpretation of R/D data from different studies. The thermal environment, the stage of the animal, and (most probably) species can all

alter the relation between R/D and growth rate or nutritional condition. For example, because fish are ectotherms the thermal environment affects a fish's metabolic rate including the activity of rRNA (Millward et al. 1973) and the rate of protein synthesis (Smith et al. 2000). Fish residing at higher temperatures have been shown to have higher growth rates per R/D value than their cold-temperature counterparts (Buckley 1982, 1984; Goolish et al. 1984; Ferguson and Danzmann 1990; Folkvord et al. 1996; Fernandez 1997; Caldarone et al. 2003). In the larval stage, protein synthesis is primarily directed toward somatic growth, whereas in later stages, an increased proportion of protein synthesis is involved in enzyme and cell turnover. It has been shown that the R/D-growth relation can change depending on the life stage investigated (Peck et al. 2003; Caldarone 2005).

Buckley (1984) developed a generalized UV-based R/D-temperature-growth model using data from 8 species of larval temperate marine fishes reared under a variety of feeding conditions and temperatures (2 to 20 °C). Other researchers have published fluorometric-based species-specific growth models (e.g., Folkvord et al. 1996; Grønkjær et al. 1997; Caldarone et al. 2003; Caldarone 2005). It is still unknown whether Buckley's model is universally applicable to all fish larvae. Differences in analytical protocols has precluded intercomparison of the models. It is hoped that the standardization method outlined in the present study will enable comparison of fish R/D-growth models developed in the future and allow researchers to determine whether a universal model or species-specific model is most accurate.

Best Practices—Because fluorometric assays are sensitive to procedural details, we present here a list of recommendations and precautions, based on the collective experience of the authors, to be considered when quantifying bulk nucleic acids in fish tissues with fluorometric-based protocols.

Sample Preparation

1. Any manipulation of the fish should be fast and precise; the sample must be maintained in as cold as possible condition to avoid degrading the nucleic acids during handling. The use of gloves is recommended.
2. The R/D value of larvae may be altered by the contents of the gut. Prior to analyzing whole larvae, consider verifying that there are no differences between total and eviscerated larvae.
3. Removing otoliths can result in a variable loss of tissue from the head region, which in turn can affect the R/D value if it is calculated on a per-larva basis. Experienced personnel can minimize this loss.
4. Different tissues and different parts of a fish possess different R/Ds (Houlihan et al. 1988; Fernandez 1997). If a fish is subsampled, it is best to determine whether the R/D values from the different subsamples are consistent within the fish before proceeding with the analyses.

5. Samples can be adequately homogenized by mechanical (e.g., tissue grinders, glass beads) and/or chemical (e.g., proteases, detergents) methods.
6. A step that will lyse the cells and dissociate proteins from the nucleic acids is necessary to facilitate binding of the fluorochrome (e.g., histones will block binding of EB to nucleic acids by as much as 40% [Morgan et al. 1979]). Adding detergents or proteinase K to tissue homogenates has proven to be effective.

Fluorescence Assay

1. Auto- (or endogenous or self-) fluorescence is the natural fluorescence of a sample with no fluorophore added. Residual fluorescence is the fluorescence remaining after treatment of a sample with both RNase and DNase. When analyzing a different species, stage, or tissue type, the presence/absence of auto- and residual fluorescence should be determined and accounted for before the protocol is routinely used.
2. Analyzing a control homogenate with each assay can be a beneficial quality-control measure. A control homogenate can be made with any surplus fish in good condition. The fish or tissues should be homogenized thoroughly, divided into vials, and frozen until use. One vial is analyzed each day along with the samples. Consistent control homogenate values verify that 1) the daily assays are accurate and 2) the nucleic acid standard concentrations are consistent from batch to batch. Alternatively, a certified nucleic acid reference material could be used for this purpose; however, at the present time, no such resource is available.
3. Fluorescence yields are very sensitive to temperature: even a 2 °C change in temperature will significantly affect yield. A constant sample temperature should be maintained when reading fluorescence values.
4. The characteristics of the compound chosen as a standard determine not only the fluorescent quantum yield upon binding to the fluorophore, but also the day-to-day and batch-to-batch variability of the response. The user should know these sources of variability when choosing standards. Given the current impossibility of purchasing nucleic acid standards that exactly match the natural samples being analyzed, highly purified eukaryotic RNA (e.g., 18S and 28S rRNA from calf liver) and DNA (from calf thymus) are good choices.
5. Storage of both samples and standards at -75 °C is recommended. Also, nucleic acids are adversely affected by repeated freezing and thawing.

Future Analytical Developments

1. The protocols in this study use nonspecific fluorophores. Ideally, fluorescent dyes will be developed

that are specific for RNA or DNA only. This will eliminate the need for treatment of samples with 1 or 2 nucleases and the possibility of incurring errors as a result of sequential subtractions.

2. Ideally, an affordable certified nucleic acid reference material (which would contain a known RNA content, DNA content, and R/D ratio) will be produced. Analyzing this material with each assay would allow for a direct numerical conversion between protocols.

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