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QUANTIFICATION OF MANILA CLAM *RUDITAPES PHILIPPINARUM* (ADAMS & REEVE, 1850) LARVAE BASED ON SYBR GREEN REAL-TIME POLYMERASE CHAIN REACTION

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ABSTRACT Biological and ecological research is seriously handicapped because of difficulties experienced in the reliable detection and quantification of bivalve larvae. This is a critical issue in the case of the Manila clam *Ruditapes philippinarum* (Adams & Reeve, 1850), a largely invasive and commercially relevant species, with important wild, cultured, and naturalized populations throughout the world. A SYBR Green real-time PCR assay, containing TPHI16S1F and TPHI16S2R primers (specific to *R. philippinarum* female mtDNA), was designed and tested to provide a rapid and high-throughput PCR-based method for larval quantification. Accurate estimations of larval numbers in spiked plankton samples point to the usefulness of this system. It can be used through a wide range of evaluated variable experimental conditions, such as the presence of closely related bivalve species, stationary-dependent plankton abundance, sampling volumes, and larval size.

KEY WORDS: Manila clam, *Ruditapes philippinarum*, 16S rRNA, real-time PCR, SYBR Green, larvae detection, larvae quantification

INTRODUCTION

The study of exploited bivalve populations is largely based on the analysis of production data, size structure, reproductive patterns, and genetic structure. These analyses involve census, recruitment monitoring and estimation of growth rate, condition index, gonadal maturation stages, spawning dates, mortality rate, and gene flow (Gosling 2003). However, studies involving crucial larval stages, both in wild and cultured populations, still remain largely inaccessible because of the difficulties experienced with identifying species reliably using microscopy, and with processing a large number of samples for quantifying planktonic larval distributions (Hare et al. 2000, Elizabeth & Cheryl 2002).

The application of rapid, species-specific, and high-throughput PCR-based methods for bivalve larvae detection have been reported only in a few cases. One example is the use of a multiplex PCR method to identify larvae from six commercially relevant species (Hare et al. 2000). Another example is the use of amplification of species-specific microsatellite alleles for identifying the European oyster, *Ostrea edulis,* larvae (Morgan & Rogers 2001). In the case of the Pacific oyster, *Crassostrea virginica*, larval detection was carried out using species-specific primers that flanked mitochondrial COX1 sequences (Patil et al. 2005). Detection of the golden mussel, *Limnoperna fortunei*, was likewise carried out using COX1 species-specific primers (Pie et al. 2006, Boeger et al. 2007). However, these methods do not provide an accurate quantification of larval number. There thus arose the need for detecting and quantifying bivalve larvae using real-time PCR as demonstrated in other marine invertebrates (Vadopalas et al. 2006). Therefore, a methodological approximation based on SYBR Green-based real-time PCR was used for quantifying the invasive golden mussel larvae (Endo et al. 2009).

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The Pacific Manila clam, *Ruditapes philippinarum* (Adams & Reeve, 1850), was originally distributed throughout the subtropical and low boreal coasts of the Indo-Pacific basin. It is currently the most commercially relevant nonautochthonous cultured clam in Europe and in other parts of the world (FAO 2005). The Manila clam's habitat adaptability, fast growth, high productivity, and disease resistance are characteristics that are quite suitable for clam aquaculture and favor survival success of accidental introductions in diverse worldwide coastal areas (Gosling 2003). This has led to the Manila clam's wide distribution outside its original range—along the northeast Pacific, North Atlantic, and Mediterranean coasts—which has given rise to naturalized populations that are now available for exploitation (Jensen et al. 2004). This species shows an unusual double uniparental inheritance (DUI) of mitochondrial DNA (mtDNA), that results in heteroplasmic F and M types of mtDNA (Passamonti & Scali 2001).

The first accidental introductions of this species took place in Hawaiian waters and along the Pacific coast of North America during the 1930s. Culture of this species was introduced in 1972 in France, and later in the United Kingdom. It was then introduced into Ireland, Portugal, Spain, and Italy to replace the problematic native (European) grooved carpet shell, *Ruditapes decussatus*, (FAO 2005). Other aquaculture initiatives spread its culture into diverse world regions and currently account for the major contribution to the annual global yield of clams. The current major culture areas in Europe are located in the Adriatic Sea (Italy), Normandy to Arcachon coast (France), and the Galician and Basque regions (Spain) (Gosling 2003). Production in the Venice Lagoon (Italy) during recent years accounts for 25,000 T (Pellizzato et al. 2010). The production of Manila clam in the Galician Rías was 1,951,499 kg in 2009 (Galicia, Spain). Production increased steadily from 2002 to a peak in 2008 at 2,261,904 kg (Xunta de Galicia 2010). In Arcachon Bay, the 2007 annual production was 1,000 T (Caill-Milly et al. 2008).

The objective of the current work is to develop a real-time PCR method to monitor effectively the presence of larval stages of the Manila clam because this step is decisive for solving critical issues on relationships between culture and naturalized populations. The method will facilitate research into factors that affect recruitment; furthermore, it will define spatial and temporal patterns of larval distribution of this largely invasive and commercially relevant species.

MATERIALS AND METHODS

Sampling

Samples ($n = 11$) of adult Manila clam, *R. philippinarum*, were captured at two locations—Ría de Arosa and Ría de Noia (Galicia, northwest Spain)—and supplied by an experimental hatchery (CIMA-Corón, Vilanova de Arousa, Galicia, Spain). A batch of *R. philippinarum* larval samples containing 2-day-old larvae (100 μm), 1 wk old (160 μm), and pediveliger stage (220 μm) was provided from laboratory cultures (CIMA-Corón). Additional samples from other cultured, exploited, and commercially relevant clam species along the Galician coast (*Venerupis senegalensis*, $n = 21$; *Tapes rhomboides*, $n = 12$; and *R. decussatus*, $n = 17$) were also acquired. A further 20 species of adult native bivalves were sampled at the same locations in the Galician Rías ($n = 1$ to 4 individuals per species; Table 1).

Environmental plankton samples (EPSs) were obtained by pumping 100 L seawater from a 1-m depth at the Ría de Noia location and filtering the same through a mesh to retain planktonic material within the 63–312 μm size range. Plankton samples were preserved in 95% ethanol and PCR tested (discussed later) for absence of *R. philippinarum* larvae. Two replicates of simulated environmental plankton samples (SEPSs) were prepared by inoculation of 1, 10, and 100 *R. philippinarum* larvae in the EPS.

TABLE 1.

List of cultured and commercially relevant clams and native bivalves commonly present in Galician waters (northwest Spain) that have been analyzed in this study.

Species	Family	*n* Sequences	GenBank Accession No.
Ruditapes philippinarum	Veneridae	11	JF901812
Ruditapes decussatus		17	JF901813
Venerupis senegalensis		21	JF901814
Tapes rhomboides		12	JF901815
Dosinia lupinus		1	JF901816
Pectunculus exoletus		1	JF901817
Venus verrucosa		2	JF901818
Venus casina		1	JF901819
Venerupis aurea		1	JF901820
Cerastoderma edule	Cardiidae	1	JF901821
Acanthocardia echinata		1	JF901822
Laevicardium crassum		1	JF901823
Aequipecten opercularis	Pectinidae	1	JF901824
Pecten maximus		1	JF901825
Mimachlamys varia		2	JF901826
Ensis ensis	Pharidae	2	JF901827
Ensis siliqua		1	JF901828
Ensis arcuatus		3	JF901829
Solen marginatus	Solenidae	1	JF901830
Gari depressa	Psammobiidae	1	JF901831
Arcopagia crassa	Tellinidae	2	JF901832
Glycymeris pilosa	Glycymerididae	1	JF901833
Spisula solida	Mactridae	4	JF901834
Mytilus galloprovincialis	Mytilidae	1	JF901835

Number (*n*) of specimens used to obtain partial 16S rRNA sequences and the GenBank accession number for each detected haplotype are provided.

DNA Isolation

All adults and larvae were preserved in 95% ethanol at –20°C. Larvae were measured, isolated, and counted under a Nikon SMZ800 binocular lens (Nikon, Kingston, UK). The adductor muscle (30 mg) from morphologically identified adults, a variable number (1–128) of whole cultured larvae (mean sizes, 220 μm, 160 μm, and 100 μm), an EPS pellet (40 mg), and spiked SEPSs were used for DNA isolation by means of the EZNA Mollusk DNA kit (Omega Bio-Tek), and were eluted in a volume of 200 μL.

Amplification and Analysis of the 16S rRNA Gene

Partial 16S rRNA sequences were amplified from all collected bivalve specimens using the 16Sa and 16Sb primers (Palumbi 1998). Amplifications were performed in a 15-μL reaction using a GenAmp 9700 thermocycler (Applied Biosystems, Foster City, CA) and containing 1× GoTaq buffer (Promega, Madison, WI), 2.5 mM $MgCl_2$, 200 μM each dNTP, 0.3 U GoTaq, 0.1 μM each primer, and 25–50 ng DNA. The PCR profile was 94°C for 3 min followed by 35 cycles of 94°C for 40 sec, 50°C for 40 sec, 72°C for 90 sec, and a final extension of 7 min at 72°C. PCR products were treated enzymatically with ExoSAP-It (GE HealthCare, Buckinghamshire, UK), and cycle sequencing was performed with the BigDye 3.1 kit (Applied Biosystems). Extension products were purified with DyeEx columns (Qiagen, Hilden, Germany), and separated electrophoretically and detected in an ABI PRISM 377XL automated sequencer (Applied Biosystems). Electropherograms were revised, and sequences were edited and aligned with BioEdit 7.0.1 (Hall 1999). A set of species-specific primers TPHI16S-1F (5′-CTGAGTTTTTAATTGAAGTT TAGTTGGG-3′) and TPHI16S-2R (5′- CCCTGCGGTAGC TTTTGCT-3′) for the *R. philippinarum* mtDNA female genome were designed using Primer Express (Applied Biosystem).

Real-Time PCR

Real-time PCR was based on the use of the Power SYBR Green PCR Master Mix (Applied Biosystems), and was performed in the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The PCR profile included a denaturation–activation 10-min step at 95°C and a single annealing–extension 1-min step at 60°C. Reactions of 25 μL volume contained 1× Power SYBR Green PCR Master Mix, a concentration of 300 nM each primer (TPHI16S-1F and TPHI16S-2R), and 2 μL DNA solution. Efficiency of PCR was calculated by means of the C_T estimation through a range of DNA concentrations (7.5 to 0.0075 ng) (Rasmussen 2001). Linearity was evaluated by amplifying DNA isolated from a variable number of larvae (1, 2, 4, 8, 16, 32, 64 and 128 larvae), including five replicates of each number. The obtained C_T values enabled calculation

of a coefficient of variation for evaluating pipetting errors as well as variability and reproducibility in the DNA isolation procedure for different samples with an identical larval number.

The inclusivity and specificity of primers were evaluated in silico through BLAST (Johnson et al. 2008), and empirically by SYBR Green amplification using the DNA template isolated from all available Manila clam and native bivalve samples.

Quantification assays were based on the standard curve method from five replicates of different DNA isolates. They were extracted from 1, 2, 4, 8, 16, 32, 64, and 128 larvae 220 μm in size. Larval number in SEPSs was estimated from two different isolates of SEPSs, each containing 1, 10, and 100 larvae, and including three reaction replicates. DNA template from SEPSs was used in a 1:10 dilution to avoid inhibition arising from an excess of total planktonic DNA in the PCR. Detection of any inhibitory effect was assessed by C_T value estimation in parallel positive controls, which included 0.5 ng target DNA template.

The effect of larval size on C_T estimation from samples containing a number of larvae was studied. Thus, each sample set of varying larval number (including 2, 8, and 32 larvae) consist of three subsamples of varying larval size (including larvae that were 100, 160, and 220 μm in size). The nonparametric Kruskal-Wallis test was applied to compare C_T values among samples that contained identical larval number but with different-size larvae.

RESULTS

A set of 11 partial 16S rRNA sequences was aligned from samples of *R. philippinarum;* in these sequences, only one female mtDNA haplotype was detected. The complete alignment included the 16S rRNA partial sequences from another three common and commercially relevant clam species (*R. decussatus*, *V. senegalensis* and *T. rhomboideus*), and other bivalve species present in Galician waters (northwest Spain; Table 1). The said alignment and additional GenBank available homologous sequences for the same species were used to design the TPHI16S-1F and TPHI16S-2R primers flanking a 116-bp fragment in *R. philippinarum*. A minimum of three species-specific polymorphisms, mainly located in the 3′- end, were selected to confer exclusivity to each primer.

The C_T values estimated from variable concentrations of template DNAs isolated from adult Manila clams ranged from 17.88–29.53; however, no signal ($C_T \geq 40$) was observed in template DNAs isolated from the remaining native bivalves and *R. philippinarum* male mtDNA. Dissociation analysis of the amplicon shows a melting temperature of about 73°C, which suggests correct amplification of a single PCR product, without primer-dimers or unspecific PCR artifacts (Fig. 1).

The PCR slope in the SYBR Green assay was –3.269 ($r^2 = 0.9906$, $P = 0$), suggesting almost 100% efficiency. Linearity was maintained through a range of 6 orders of magnitude, from the 1% of a DNA solution volume isolated from 128 larvae to the 0.00001%, representing a dynamic range from 30 ng–0.3 pg DNA (Fig. 2). A DNA concentration in reaction above 30 ng showed an inhibitory effect on PCR; consequently, an appropriate template dilution was carefully considered because EPSs provide 15–35 ng/μL. SD, related exclusively to pipetting error increased proportionally with decreasing template DNA, and ranged from 0.074–0.448 (mean, 0.288).

Linearity was also observed for C_T values estimated using the 1% DNA solution volume isolated from 1–128 larvae ($r^2 = 0.9246$, $P = 0$) as a template. Mean C_T values are often outside the 95% confidence interval for the extreme values (1 larva and 128 larvae), and their SDs are higher (Fig. 3).

The quantification assay of SEPSs allowed a reasonable estimation of the spiked number of 220-μm larvae, even using undiluted standards and 10-fold diluted SEPSs. After adjusting for the 10 dilution factor, the estimated mean larval number was 1.20 ± 0.026, 14.9 ± 0.382, and 100.4 ± 1.711 for SEPSs spiked with 1, 10, and 100 larvae, respectively (Fig. 4). The 1-sample *t*-test suggested that differences between the estimated numbers

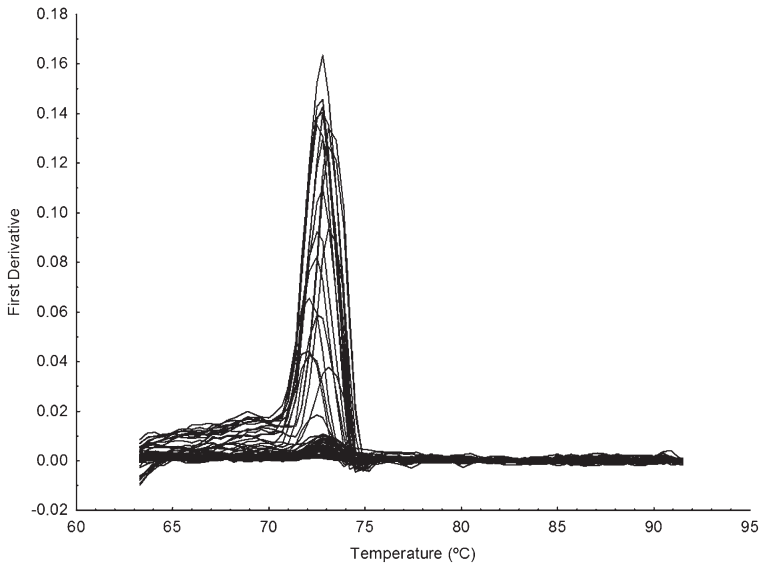

Figure 1. Amplicon dissociation curve generated by using DNA from different specimens of Manila clam, *R. philippinarum*, as a template, with TPHI16S1F/TPHI162R primers in the SYBR Green assay.

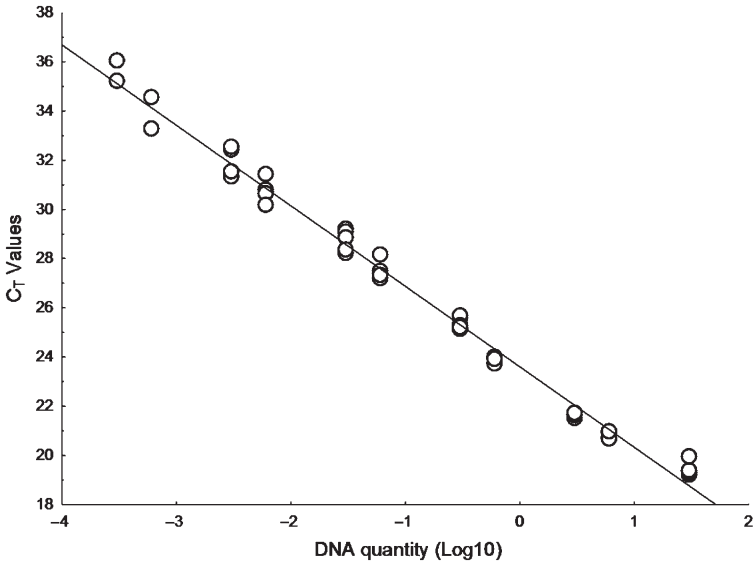

Figure 2. Relationship between log10 of *R. philippinarum* DNA (ng) and the estimated C_T value in the SYBR Green real-time PCR assay using TPHI16S1F/TPHI162R primers. $r^2 = 0.9906$; $P = 0$; $y = 23.6036 - 3.2693 \times x$.

and the spiked numbers were nonsignificant ($P < 0.05$) Similar accuracy was obtained using the undiluted template from the same SEPSs.

The estimated C_T value for the different number of larvae changed (2, 8, and 32) with larval size. When 1% DNA isolated from two larvae was used as template, the C_T value was similar in the case of DNA isolated from 160- and 220-µm larvae (26.34 ± 0.12 and 26.59 ± 0.16, respectively). However, the C_T value estimated for the 100-µm larva was higher (27.40 ± 0.24; Fig. 5). The same relationship of C_T values was observed for each of the assayed larval numbers, although the C_T difference is greater (>2 cycles) when considering the 8 larvae and 32 larvae. Thus, the nonparametric Kruskal-Wallis test suggests that when comparing C_T values from the three larval sizes, the 100-µm C_T value is significantly different from the 160- and 220-µm larval size C_T values ($P < 0.05$), except when compared with 220-µm C_T data included in the 32 larvae data set ($P = 0.14$). Moreover, 160- and 220-µm data showed no significant difference in their C_T values for the 2, 8 ($P = 1$), and 32 ($P = 0.07$) larval number data sets.

DISCUSSION

The lack of an efficient methodology to detect and quantify species-specific bivalve larvae hinders researchers from carrying out diverse biological and ecological studies that are focused on this critical stage. The SYBR Green system (TPHI16S-1F

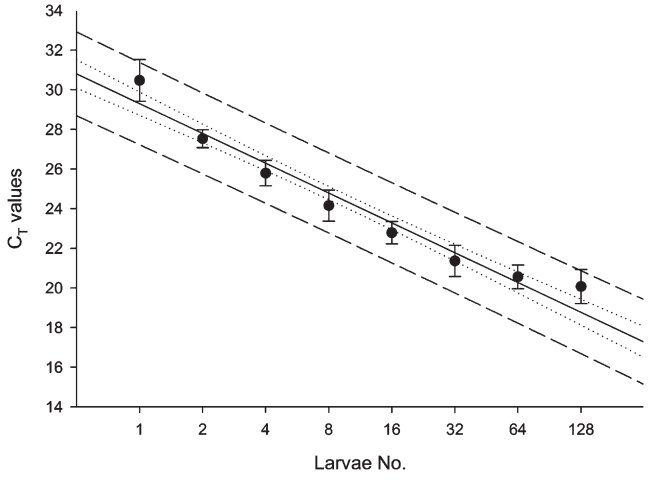

Figure 3. Relationship between larval number of *R. philippinarum* and the estimated C_T values from real-time PCR. Black dots are mean values from 5 DNA isolates; error bars are SDs. The solid line corresponds to the linear regression ($r^2 = 0.9246$; $P = 0$; $y = 29.2932 - 4.9961$). Confidence intervals and prediction intervals (95%) are depicted with dotted and dashed lines, respectively.

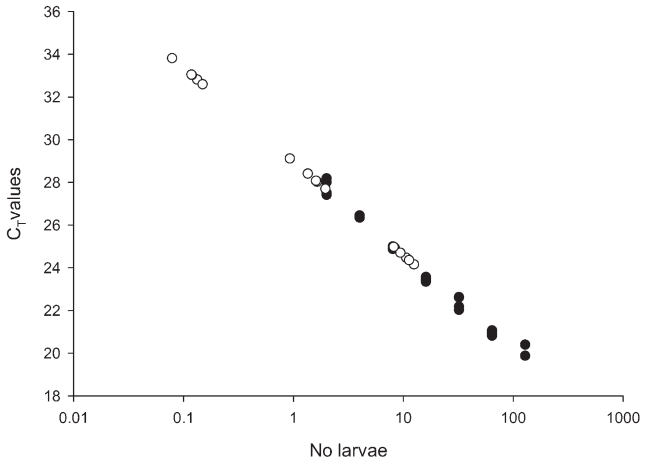

Figure 4. Plot of the relationship between larval number and the estimated C_T values. Black points correspond to values estimated for larval number standards (1, 2, 4, 8, 16, 32, 64 and 128 larvae; $R^2 = 0.989$), whereas white points refer to estimated values for a 1:10 dilution of spiked environmental plankton samples (1, 10, and 100 larvae).

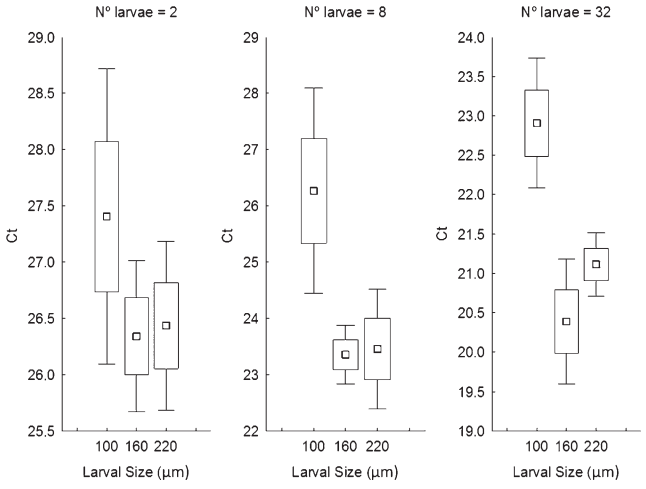

Figure 5. Variation in C_T values observed for 3 sets of different number of *R. philippinarum* larvae (2, 8, and 32), each including three 3 larval sizes (100, 160, and 220 µm). Square points are the mean; bars are the mean ± SD and lines are the mean ± 1.96 × SD.

and TPHI16S-2R primers) was brought into consideration to solve such deficiency in the case of the ecologically and commercially relevant Manila clam, *R. philippinarum*. The accurate estimation of larval number in spiked plankton samples suggests the usefulness of this system for a wide range of variable experimental conditions, such as the presence of closely related bivalve species, stationary-dependent plankton abundance, sampling volumes, and larval size.

The detection of a single haplotype in the local sample (Galician Rías) of *R. philippinarum* reflects the low intraspecific variability in this conserved ribosomal domain. It is the absence of polymorphism within the primer sequences that guarantees the successful design of a detection system, which thus provides inclusivity. Conversely, this ribosomal sequence carries out gender and species-specific polymorphisms, thereby conferring exclusivity to this detection system. No signal ($C_T \geq 40$) in reactions was consequently observed for other bivalve species and male mtDNA of *R. philippinarum*.

Total amount of DNA used as a template in any quantification experiment is limited by the inhibitory effect of a high DNA concentration in PCR. Such inhibition is recurrently observed in similar studies (Pan et al. 2008). The SYBR Green–based reaction allowed a detection of 0.3 pg total DNA, which is about 3 orders of magnitude below the DNA used as a template from a single larva. This sensitivity permitted the use of highly diluted DNA templates in the assay, thus avoiding the effect of PCR inhibitors and DNA isolation and elution from complex and rich-planktonic matrices, in increased volumes.

An EPS provides not only DNA isolated from the target larvae, but also variable concentrations of DNA from other plankton species. Thus, a 1:10 dilution of DNA isolates from SEPS was used to avoid PCR inhibition under these sampling conditions. The detection limit allows for the use of a wide range of sampling volume of seawater (lesser or greater than the volume used here: ~500 L), according to sampling capacities and plankton abundance. This approach provided an accurate estimation of the spiked larvae in SEPSs, even by extrapolation beyond the dynamic range of used standards.

A linear relationship between C_T values and the number of larvae is required to estimate the unknown environmental sample larval number using the standard curve method. The observed SDs are likely explained by (1) differential efficiency of the DNA isolation method from samples containing varying larvae number, (2) minor PCR inhibition in reactions with high template concentration, and (3) pipetting errors. These two latter issues could be involved congruently in C_T deviations observed for increasing DNA concentrations (Fig. 3).

The amount of mtDNA molecules per cell shows an important variation depending on cell type, but is observed to be constant within each type (Robin & Wong 1988). The use of total DNA from complete larvae obviates this variation. Therefore, quantification is based on the fine correlation of the number of mtDNA genomes with the cell number. Consequently, the same number of larvae from different developmental stages will yield different C_T values. If so, then estimations based on standards prepared with a given larval size will yield inconsistent values for environmental samples containing different larval sizes. Values are therefore related to cell numbers and not larval number. However, the quantification assay of pinto abalone species *Haliotis kamtschatkana* suggests an absence of any detectable effect of larval size on larval number estimates (Vadopalas et al. 2006). Our experience with C_T values observed for a variable number (2, 8, and 32) of *R. philippinarum* larval sizes (100, 160, and 220 µm) nevertheless shows a relationship between larval size and C_T. Thus, C_T estimates for the same larval number of 100-µm larvae are significantly higher than those for 160- and 220-µm larvae. Such size-dependent C_T values can consequently lead to underestimates of the number of 100-µm larvae. This situation can be avoided by estimating mean size of sampled larvae using a lens. Alternatively, the adequate and homogeneous EPS must be obtained by size-selecting filtration (Vadopalas et al. 2006). In both cases, samples are then quantified using an appropriate-size standard. For instance, the number of Manila clam larvae, with a size range from 160–220 µm, can be estimated correctly from a standard curve based on a 220-µm size standard. In like manner, any identical C_T values estimated from 160–220-µm larval sizes are the result of an undistinguishable variation in the mitochondrion-per-cell number or a loss of correlation between tissue development and measured shell size.

The application of this real-time PCR methodology is currently focused on the study of larval abundance and distribution patterns for this cultured and invasive species in Europe. This information is of interest for culture management and ecological control, mainly in areas such as in the ecologically sensitive Galician Rías, which are devoted to aquaculture of native bivalve species.

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