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Diplolaimella dievengatensis (Nematoda: Monhysteridae)
como organismo modelo em ensaios de ecotoxicidade

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Diplolaimella dievengatensis (Nematoda: Monhysteridae) as model
organism in ecotoxicity assay

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Resumo

Nematodos marinhos de vida livre são o mais onipresentes, abundantes e diversos componentes da meiofauna em comunidades bêmicas. São excelentes organismos modelo devido a seu curto ciclo de vida, ampla disponibilidade e viabilidade de cultivo com mínima estrutura laboratorial. Neste estudo, uma população de *Diplolaimella dievengatensis* Jacobs 1991 (Nematoda, Monhysteridae) do estuário relativamente prístino do rio Guaratuba, São Paulo, Brasil foi isolada e cultivada. Os objetivos foram: *i*- aplicar uma abordagem taxonômica integrativa a fim de comparar esta população do Brasil com outra da localidade tipo dessa espécie, da costa da Bélgica, com relação à dados morfológicos, do ciclo de vida e molecular do gene 18S do rDNA; e *ii*-comparar, à luz da teoria da história de vida, repostas de parâmetros do ciclo de vida tais como: fecundidade, crescimento e sobrevivência de *D. dievengatensis* (aqui como espécie mais lenta) e *Litoditis marina* (Bastian, 1865) (Nematoda, Rhabditidae) (como espécie rápida) sob exposição subletal ao surfactante dodecil sulfato de sódio (DSS). A população do Brasil foi similar a da costa da Bélgica em todos os parâmetros. Embora análises morfométricas consideraram *D. dievengatensis* do Brasil isometricamente maior que a população belga em relação em algumas características, a presença de todos os caracteres diagnósticos confirmaram a similaridade de ambas as espécies. Os parâmetros de ciclo de vida, tempo de eclosão, comprimento corporal final e biomassa foram similares em ambas as populações, nas quais fêmeas foram maiores que machos. Crescimento populacional, mensurado como taxa intrínseca de crescimento natural foi ligeiramente mais alta para *D. dievengatensis* do Brasil ($r_m=0.41$), que para a população europeia ($r_m=0.348$). Comparações moleculares no Genbank mostraram 99.4% de similaridade entre ambas populações, indicando portanto que a população do Brasil é similar a *D. dievengatensis* belga. No capítulo *ii*, ambas as espécies responderam distintamente a exposição ao SDS. Supreendentemente as taxas de crescimento e de reprodução de *D. dievengatensis* foram incrementadas sob concentrações mais baixas e intermediárias (0.001 e 0.003%), enquanto para *L. marina* esses parâmetros foram reduzidos em todas as concentrações de DSS testadas (0.001, 0.003 e 0.006%). O DSS não afetou a sobrevivência de adultos da espécie mais lenta. Por outro lado, a sobrevivência de adultos da espécie mais rápida foi significativamente afetada pelo SDS e esse efeito foi dependente do sexo, com taxas reduzidas em machos expostos a 0.006 % de DSS. Embora ambas as espécies estejam proximamente dispostas ao longo do gradiente “rápido-lento”, elas responderam diferentemente ao efeito tóxico do DSS. Efeitos sobre *L. marina* se enquadram no padrão de espécies mais rápidas ao alocar menores investimentos para as defesas contra danos fisiológicos e para sua própria manutenção somática. Nós propomos que a aparente menor resistência desta espécie rápida sob estresse ao nível individual é balanceada por suas altas taxas reprodutivas, conferindo mais alta resiliência a essa espécie ao nível populacional. Por fim, foi demonstrado que *D. dievengatensis* é um importante organismo modelo nativo que pode ser usado em uma ampla diversidade estudos e testes experimentais. Identificar e compreender diferentes efeitos do estresse dentro do contexto da teoria da história de vida é um aspecto importante, o qual tem aumentado nosso conhecimento sobre as ameaças de atividades antropogênicas sobre comunidades naturais.

Palavras-chave: Taxonomia integrativa, nematodos marinhos, ecotoxicologia, teoria da história de vida, DSS

Abstract

Free-living marine nematodes are the most ubiquitous, abundant and diverse meiofaunal component of benthic communities. These are excellent model organisms, due to its short life span, wide availability and feasibility to cultivate with minimum laboratory facilities. In this study, a population of *Diplolaimella dievengatensis* Jacobs 1991 (Nematoda, Monhysteridae) from the relatively Pristine estuary of the Guaratuba River in São Paulo, Brazil was isolated and cultivated. The goals were; *i*- to apply an integrative taxonomic approach in order to compare this population from Brazil with another from the species type-locality in the Belgian Coast, with regard to morphological, life-cycle and the 18S gene of the rDNA molecular data; and *ii*- to compare, at the light of life history theory, the responses of life history parameters such as; fecundity, growth and survivorship of *D. dievengatensis* (here as a slower species) and *Litoditis marina* (Bastian, 1865) (Nematoda, Rhabditidae) (as a faster species) under sublethal exposition to the sodium dodecyl sulfate (SDS) surfactant. The population from Brazil was similar to that from Belgium coast in all parameters. Although morphometric analyses considered the Brazilian *D. dievengatensis* isometrically larger than the Belgian population regarding some characters, the presence of all diagnostic characters confirmed the similarity of both species. The life-cycle, hatching time, final body length, and biomass parameters were similar in both populations, in which females were larger than males. The population growth, measured as intrinsic rate of natural increase was slightly higher for the *D. dievengatensis* from Brazil ($r_m=0.41$), than the European population ($r_m=0.348$). Molecular comparison on Genbank showed 99.4% of similarity between both populations, indicating therefore, that *D. dievengatensis* from Brazil is similar to those from Belgium. In the chapter *ii*, both species responded differently to SDS exposition. Growth and reproduction rate of *D. dievengatensis* surprisingly were enhanced at low and intermediate concentrations of SDS (0.001 and 0.003%), while for *L. marina* these parameters were reduced in all SDS concentrations tested (0.001, 0.003 and 0.006%). The SDS did not affect the survivorship of adults of the slower specie. On the other hand, survivorship of adults of the fast species was significantly affected by SDS and this effect was dependent on adult gender, with reduced rates of males exposed to 0.006% SDS. Although both species are located nearby along the fast-slow continuum, they responded distinctly to of the toxic SDS effect. Effects over *L. marina* met the trend of faster species in allocating fewer investments in defenses against physiological injuries and on their own somatic maintenance. We propose that the apparent lower resistance of this faster species under stress at the individual level is balanced by their higher reproductive rates, conferring higher resilience to this species at the population level. Finally, it was demonstrated that the marine nematode *D. dievengatensis* is an important native model organism which can be used in a wide range of studies and experimental tests. Identifying and understanding differential effects of stress in the context of life-history theory is an important aspect which enhanced our understanding about the threats posed by anthropogenic activities on natural communities.

Key-words: Integrative taxonomy, marine nematodes, ecotoxicology, life-history theory, SDS

GENERAL INTRODUCTION

Nematodes are one of the most abundant and diversified groups in the animal kingdom (TAHSEEN, 2012), and found in nearly all environments, such as terrestrial, freshwater and marine habitats (HOLME & MCINTYRE, 1971). There are over 26,000 described nematode species (VENEKEY et al. 2010), although estimates of the real diversity of this group ranges from one up to one hundred million (LAMNSHEAD et al. 1993). For aquatic systems, nematodes are considered to be the most abundant metazoans living in sediments, with densities that can be around one million individuals per square meter (HEIP et al., 1985). These occur through all marine and estuarine substrates, from the intertidal zone up to the oceanic trenches, and the number of species found is usually one order of magnitude larger than any other taxa (LAMBSHEAD et al. 1993). There are four to five thousand known species in these environments, which is again, probably far below the real diversity of this group (VENEKEY et al. 2010). Despite the omnipresence of nematodes in a wide diversity of ecosystems, studies about these organisms were developed mainly due to the significant importance of disease-causing nematodes for agriculture and human health (CHITWOOD and MURPHY, 1964; WILSON, 1976). While the interest in parasitic nematodes has continuously grown, attention to free-living aquatic and terrestrial nematodes from different environments is still modest (FERRIS; 1995). Therefore, marine nematodes are far less known regarding questions of their biology, diversity, ecology, and function compared to their pathogenic counterparts.

In 1974, however, the complete characterization of the genome of *Caenorhabditis elegans*, represented an important landmark concerning the study of the biology of free-living nematodes (BRENNER, 1974). Since then, this species has become a model organism for diverse fields, especially in genetics and developmental and molecular biology (RIDDLE, 1980; STRINGHAM and CANDIDO, 1994), and opened a new perspective in the study of nematode biology. Despite the enormous contributions, for ecological studies *C. elegans* do not represent an ideal model organism, since nematodes can have a wide functional diversity and great variety of life history strategies and habitats. Particularly for environmental impact studies, a model organisms should ideally be a native species, which represent the local environment (FREITAS and ROCHA, 2012).

Mainly in the 70's and 80's decades, efforts were applied to culture marine and estuarine nematodes. Several species were isolated, cultivated and used in experiments (TIETJEN et al., 1970; HOPPER et al, 1973; VRANKEN et al., 1981; WARWICK, 1981; VRANKEN and HEIP, 1983; VRANKEN, 1987; VRANKEN et al., 1988). These cultures provided important information about the biology and ecological physiology of different species that was hitherto unknown. By means of such culture, it is possible to follow and quantify innumerable life cycle parameters, such as fecundity, egg viability, body growth rates, generation times, and mortality rates. In addition, since developmental conditions (*e.g.* temperature, salinity, food quality and abundance) can be controlled and manipulated, it also is possible to test species environmental performances in diverse conditions.

Although innumerable species could be potentially cultivated, few of them are kept and used in experimental studies. Considering both marine and estuarine ecosystems, only about 30 nematode species have ever been cultivated, more than half belonging to Monhysteridae de Man, 1876 (MOENS and VINCX, 1998). Species of this family provide useful biomonitoring tools, since they respond in distinct ways to environmental variation (GERLACH and SCHRAGE, 1971; VRANKEN et al., 1988). In some circumstances, for instance, they may exhibit dominance in ecosystems under higher nutrient levels (RISTAU et al., 2015), whereas others species were negatively correlated with pollutants (*e.g.* Pb) (HEININGER et al., 2007). However, the use of non-native species in experimental studies limits the local applicability of such investigations in regard to ecosystem functioning, as well as the effects of anthropogenic stressors to these communities. Therefore, it is paramount to expand the set of cultivated nematodes, in order to increase the representability of distinct environments and under diverse conditions.

Among the main features that compose a good model organism, stand out its wide availability and feasibility of being kept with minimum laboratory facilities. Species considered cosmopolitan or with worldwide distribution can be useful in this sense, but in many cases, the lack of comparisons among distinct locations and uncertainties with regard to correct taxonomic identification can be a problem. Since free-living marine nematodes have a set of life cycle traits which may suggest low dispersal capabilities, it is necessary to investigate whether species with wide distribution ranges represent, indeed, complexes of cryptic species (Giere, 1993). Considerable cryptic diversity has already been observed in a wide range of nematode

species (DERYCKE et al., 2013; RISTAU et al., 2015). Such question can be addressed by means of integrative approaches, which consists in using different data (morphological, developmental, molecular, behavioral, ecological, etc.), to increase the trustworthiness of species delimitation (DAYRAT, 2005).

In the chapter I, a monysterid species preliminarily identified as *Diplolaimella dievengatensis* was isolated and cultivated from a relatively pristine environment in the southeast coast of Brazil (SP). This species had never been recorded in this country before. An integrative taxonomic approach was applied in order to compare the population of *D. dievengatensis* from Brazil, with data from its type locality in the Belgium coast. The morphometry, individual and population growth parameters, and the 18S gene of the rDNA were evaluated. We expected that the Brazilian population of *Diplolaimella* to represent a distinct species within the genus, considering that its limited dispersal capabilities may result in isolation of populations from distinct sides of the Atlantic Ocean.

A detailed knowledge on life-history traits of *D. dievengatensis* was obtained and described in chapter one providing a baseline which could be applied in manipulative experiments. These traits indicate distinct life history strategies among species, which demonstrated how these develop and overcome challenges imposed by their environments. This can be applied, for instance, in order to identify and understand differential effects of chronic stress posed by anthropogenic activities. In chapter II, we investigate how two nematodes species (*D. dievengatensis* and *Litoditis marina*) dealt with stress imposed by sublethal exposition to sodium dodecyl sulfate (SDS), a detergent used in a wide range of household products. It was hypothesized that the responses would be dependent on the species' life history strategies, which can be ranked along a "fast-slow continuum" concept based on their relative investment in body size, lifespan and fecundity (ZERA and HARSHMAN, 2001; RICKLEFS and WIKELSKI, 2002). The *D. dievengatensis* was considered in this study as a relatively slower species if compared with *L. marina*. In this way, it was expected that under stress, *D. dievengatensis* prioritize self-maintenance and survival, at the expense of reproduction. Therefore individual growth and survivorship rates of *D. dievengatensis* would be less impacted by the exposition. On the other hand, since *L. marina* prioritized reproduction, it was expected that growth rates and survivorship would be more affected by the SDS.

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CAPÍTULO I

Comparison of *Diplolaimella dievengatensis* (Nematode: Monhysteridae) from Brazil
and Belgium Coast under integrative taxonomic approach

Comparison of *Diplolaimella dievengatensis* (Nematode: Monhysteridae) from Brazil and Belgium Coast under integrative taxonomic approach.

ABSTRACT

A species of the genus *Diplolaimella* from Brazil was isolated and cultivated in laboratory under constant conditions of temperature (25°C) and salinity (20), similar to its local environment. This species was previously identified as *Diplolaimella dievengatensis*, which was described for the Belgian Coast. We hypothesized that the Brazilian *D. dievengatensis* represented a distinct species within this genus and both populations were compared by means of an integrative taxonomic approach. Parameters of morphometry, life-cycle, and the 18S gene of the rDNA were analyzed. This approach demonstrated that the Brazilian species was similar to the *D. dievengatensis* from Belgium coast. The *D. dievengatensis* from Brazil had 10 morphometric parameters isometrically larger than the Belgian population, among 32 compared. Regarding to the life-cycle parameters, hatching time ($3.3 \text{ days} \pm 0.5$), final body length, and biomass were similar in both populations, in which females were larger than males. Although the *D. dievengatensis* from Brazil had a linear and continuous pattern of somatic growth, exponential growth pattern was observed in the juveniles of the European *D. dievengatensis*. The parameter of population growth, measured as intrinsic rate of natural increase was higher for the *D. dievengatensis* from Brazil ($r_m=0.41$), than the European population ($r_m=0.348$), probably due the higher generation time (14 days) in the last one, compared to the Brazilian population (10.3 days). Molecular comparison on Genbank showed 99.4% of similarity between both populations. Although geographically separated, both populations had similar life cycle patterns under laboratory condition. This similarity in life cycle parameters, the viability of cultures and wide distribution ranges the different populations appoint *D. dievengatensis* a useful model species for experimental studies.

Keywords: free-living nematodes, *Diplolaimella dievengatensis*, life-cycle parameters, integrative taxonomic, morphometry, life-cycle, 18S gene of the rDNA.

INTRODUCTION

Free-living nematodes are an important fraction of the meiofauna representing the most abundant and species-rich taxon in marine and estuarine sediments (Tom Moens *et al.*, 2013). They reach densities up to several million individuals.m⁻² and a corresponding biomass up to 10 g C.m⁻² (Heip *et al.*, 1985). In addition to their numerical importance, nematodes participate in different ecological processes such as organic matter decomposition (John H Tietjen, 1967; Koop *et al.*, 1982; D. Leduc & Probert, 2009) sediment bioturbation, which influences diffusion coefficients of various solutes (Alkemade *et al.*, 1992; Aller & Aller, 1992; Bonaglia *et al.*, 2014) and energy transfer, serving as food for higher trophic levels (Coull, 1999; Danovaro *et al.*, 2007; Daniel Leduc *et al.*, 2016). Therefore, they play a significant role in sedimentary ecosystems (Schratzberger & Ingels, 2016). Despite their high ecological relevance, the knowledge of the basic biology and ecology of free-living marine and estuarine nematodes remains limited to a few model species representing a small number of environments.

Many marine and estuarine nematodes have life cycles of few days or weeks, except for a few large predatory species (C. Heip *et al.*, 1978; T. Moens & Vincx, 1998). As result, life-history parameters, such as body growth, reproduction and survival, can be easily followed and experimentally quantified. This allows testing for the influence of many environmental factors (biotic and abiotic) over those parameters, for which laboratory culture systems of free-living nematodes offer numerous possibilities of studies. For instance, it is possible to investigate the influence of feeding regime on the fitness of species or the effects of toxic stress on individual and population development (G. Vranken *et al.*, 1981, 1988, 1991; dos Santos *et al.*, 2008).

Eleven marine and estuarine species have been isolated and cultivated (T. Moens & Vincx, 1998) but just a few of them have been used in laboratory experiments. Some of them, such as *Litoditis marina* and some species of the genus *Diplolaimella*, are considered cosmopolitans or can be found over large distribution ranges (Gerlach and Riemann, 1973; Tietjen *et al.*, 1970; Hopper *et al.*, 1973; Pastor de Ward & Lo Russo, 2009). However, it is unknown whether distinct populations share similar functional and life history parameters. Geographically separated populations are exposed to distinct environmental pressures and may exhibit phenotypic plasticity, manifested on morphological and functional (e.g. life-cycle) features (Whitman & Agrawal, 2009). It can be further expected that geographically and environmentally isolated populations

may present different genetic structures due to limited or absent gene flow between them (Slatyer *et al.*, 2013). Evidences of cryptic species are well reported for marine nematodes (Derycke *et al.*, 2005, 2012, 2013; Fonseca *et al.*, 2008; Pereira *et al.*, 2010) probably related to limited dispersal capabilities, local adaptation and short life-span.

In this study, we analyzed a species of the genus *Diplolaimella* from Brazil under an integrative taxonomy approach. This approach consists of using different data (morphological, molecular, developmental, behavioral, ecological, etc.), to increase the trustworthiness of species delimitation, since criteria used in methodologies to obtain some of these data can be variable (Dayrat, 2005; Fonseca *et al.*, 2008). The studies using the integrative taxonomy for nematodes, as well as for other metazoa, has been employed to discover cryptic species, to detail descriptions of new species or to redescribe known species (S. Derycke *et al.*, 2008; Sofie Derycke *et al.*, 2010; Oliveira *et al.*, 2012; Tchesuno *et al.*, 2014). We have compared the Brazilian species with its congeneric by means of morphometry, individual and population growth parameters, and the 18S gene of the rDNA. We expected the Brazilian population of *Diplolaimella* to represent a distinct species within the genus.

MATERIAL AND METHODS

Nematode culture in the laboratory

The *Diplolaimella* sp. specimens were isolated from sediments from the Guaratuba river estuary, situated at São Paulo coast, Brazil (23°45'53"S, 45°53'48"W), and established in monospecific, agnotobiotic cultures. More detailed outline of culture procedures for free-living marine and brackish-water nematodes is given in Moens & Vincx, 1998. Briefly, small samples of untreated sediment were inoculated in excavations made in layers of 0.6 % bacto-agar and nutri-agar solid medium (DIFCO) prepared with artificial seawater (ASW, Dietrich and Kalle, 1957) and salinity 20 (i.e. field conditions) in 9cm diameter Petri dishes. After a few days, a variable fauna and microflora arose from the inoculated material and colonized the surrounding agar. Patches of *Diplolaimella* sp. were then isolated and replicated to create gnotobiotic culture plates (Koenning & Barker, 1985).

Measurements

The morphological analysis first occurred through comparison of characters and some measures of our specimens observed under optical microscopy with the pictures and key for *Diplolaimella* presented by Fonseca & Decraemer (2008). Once this species was preliminarily identified as *Diplolaimella dievengatensis*, a more complete morphometry was made to confirm the identification from Belgian animals described by Jacobs *et al* (1990). In total, 32 animals were measured: 10 males, 10 females, three juveniles on J1 stage, two on J2, three on J3 and three on J4, all mounted in paraffin/glycerin slides. The measurements were obtained from images using the image analysis software ImageJ (NIH) and are expressed in micrometers. In order to check if there would be a separation between the Brazilian and Belgian subpopulations, mean values of the morphometric characters of males, females and juveniles, were first submitted to individual correlation analysis to select the ten most independent measurements. These selected measurements of both subpopulations were then submitted to Principal Component Analysis (PCA), using the R packages STATS and ggbiplot (Everitt & Hothorn, 2010) .

Life cycle and population growth parameters

Several gravid females of *Diplolaimella* sp. from monoxenic cultures in exponential growth were moved to new agar plates before laying the eggs in order to have synchronized worm cultures. After 24h, the laid eggs were transferred to fresh plates with bacto-agar medium (DIFCO) 0.6%, 20 salinity and incubated at 25°C. Frozen-and-thawed suspensions of the bacterial strain *Escherichia coli* ATCC 11229 were used as food source at densities of 5×10^8 cells ml⁻¹, which are the optimal concentrations for *D. dievengatensis* (Moens *et al.*, 1999). The experiment started with approximately 210 eggs, divided into three plates. Measurements of the eggs were taken from the larger length and width. The hatching rate was observed and measured as the proportion of hatched eggs from egg laying to the first appearance of juveniles. After hatching, the somatic growth (body length) was measured daily until the tenth day and from then on, every two days up to twenty-eighth days. Body weight (µg wet weight) was calculated following Andrassy's (1956) formula $(LW^2/1.7)NRd.10^{-6}$, where L = total length (µm), W = width of maximum body diameter avoiding vulva in female and NRd = nematode relative density, estimated at 1.13 for marine nematodes (Somerfield & Warwick, 2013). The nematodes were photographed under a Nikon TE300 inverted

microscope equipped with a Nikon D300 camera and the pictures were analyzed using the ImageJ software (NIH). The population growth rate was measured calculating the intrinsic rate of natural increase (r_m) which is defined as the rate of increase per individual in the absence of adverse conditions (Caughley & Birch, 1971; Sibly, 1999). The life history parameters to r_m calculations were based on Euler-Lotka equation ($\sum_x e^{-r_m x} l_x m_x = 1$) where: x = time (days), l_x = age-specific survival probability, and m_x = age-specific fecundity. Growth was followed daily by 10 days where juveniles and adults were counted using a stereoscopic microscope. Number of offspring was estimated as egg and juvenile production per female. Counting was interrupted as soon as the first new adults arose in a culture. Population doubling time (PDT), which is the time needed for a growing population to duplicate (Lampert et al, 1997), was calculated using the formula: $PDT = \ln 2 / r_m$. The generation time was calculated using three alternative equations (T_0 , T_1 and T) (G Vranken & Heip, 1983). T_0 is the mean age at reproduction of a cohort of females, which formulae is $T_0 = (1/R_0) \sum_x l_x m_x$; T_1 refers to period of time necessary for a population growing at a constant rate r_m to increase by the factor R_0 , the formulae is $T_1 = (\ln R_0) / r_m$; and T is the mean age of the mothers of a set of newborn individuals in a population with a stable age distribution, which formulae is $T = \sum_x e^{-r_m x} l_x m_x$ (Addis *et al.*, 2015).

Molecular analyses

An adult specimen was picked up from culture and rinsed three times in sterile distilled water to remove compounds of the DESS solution from their tissues (Yoder *et al.*, 2006). The specimen was then transferred to 20 μ l lysis buffer (50 mM KCl, 10 mM Tris pH 8,3; 2,5 mM MgCl₂; 0,45% NP40; 0,45% Tween20) and frozen at -20°C . Next, 1 μ l of proteinase K (60 $\mu\text{g ml}^{-1}$) was added and sample was incubated for 1 h at 65°C , followed by 10 min at 95°C . Finally, the DNA sample was centrifuged for 1 min at maximum speed (14 000 rpm). The primer set G18S-18P was used to amplify the small ribosomal subunit (18S rDNA) [see (Da Silva *et al.*, 2010)] for detailed information about position, sequence, and reference of primers]. Reaction was performed in total volumes of 25 μ l containing 12,5 μ l of TopTaq Master Mix Kit Qiagen, 2 μ l of MgCl₂ 25 mM, 0.25 μ l of each primer (25 nM), 8 μ l Milli-Q water and 2 μ l DNA. Cycling conditions for the amplification were: 94°C , 5'–35x: 94°C , 30''; 55°C , 30''; 72°C , 2'– 72°C , 10'. After PCR reaction, we loaded 4 μ l of PCR product onto a 1% agarose gel to check the size of the amplified product. PCR product was enzymatically cleaned with

alkaline phosphatase (1 U/ml, Fermentas) and exonuclease I (20 U/ml, Fermentas) for 15 min at 37°C followed by 15 min at 85°C. Besides SSUG18S and SSU18P, the additional internal primers SSUA, SSU9FX, SSU9F, 24F1, SSU9R, Nem 18SR, and SSU13R were used for sequencing [see (Meldal *et al.*, 2007; Silva *et al.*, 2010) for supplementary information]. Sequencing was performed on an ABI PRISM 3100 Genetic Analyzer, after the amplification of purified sample with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) under the following conditions: an initial denaturation of 2 min at 98 °C was followed by 40 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 5 s and extension at 60 °C for 60 s. Complementary strands were combined, edited and then, aligned with sequence of *D. dievengatensis* (Genbank AJ96682.1) using Geneious Pro v5.6.5 (<http://www.geneious.com/>, Kearse *et al.* 2012).

RESULTS

Measurements

The animals of this study were quite similar to those first described by (Jacobs *et al.*, 1990). A description of Brazilian specimens is presented below. Description: Males: animals have a slender body, mean of 1343.58 µm, and the diameter varying little in its extension (maximum of 25.83 µm) (Table 1). Slightly striated cuticle, forming a lip at anterior end. Lips not set off and head about 20 µm, round to slightly truncated. Cephalic arrangement difficult to view. The buccal cavity is narrow and conspicuously divided in two cuticularized chambers; the anterior chamber is funnel-shaped with a distal narrowing, and the second varies from a circular to a triangular shape; teeth not seen. Circular amphidial fovea, aperture diameter about 5 µm. Presence of one pair of ocelli, in dorsolateral and approximately half the distance from the anterior end to the nerve ring position, and bright aspect. Pharynx about 200 µm, cylindrical, muscular and lacking bulbus. Cardia rhomboid. Ventral gland not seen. One outstretched testis and right to the intestine. One pre-cloacal supplement with centrally papillae. Simple, sclerotized, arcuate about 40 µm. The gubernaculum is complex, well cuticularized, with a spicules dorsal-caudal apophysis. Presence of postcloacal papillae. The tail is mainly long and filiform, but conical in its most proximal part. Presence of three caudal glands and conspicuous spinneret. Females: body shape similar to males, but longer in length (1706.44 µm) and width (37.61 µm). Tail longer than the male tail (472.64 µm

and 335.98 μm respectively), especially the conical part. Reproductive system monodelphic, outstretched, directed anteriorly and right to the intestine ovary. The vagina is straight and well cuticularized. Vulva located about the mid-body region (about $V = 48.68\%$), subtly towards the tail. The other characters resemble those of males. Juveniles: morphology similar to adults. In general, the body becomes thicker (46.17 in J1 to 39.63 in J4), while the pharynx length becomes smaller (4.52 in J1 to 5.89 in J4), and tail length almost isometric with body length except for J2 ($c = 3.77$). Head length and amphidial fovea aperture diameter were similar throughout ontogeny.

The PCA revealed a general separation between Brazilian and Belgian specimens for males, females and juveniles (Fig. 2, 3 and 4). The figures indicate the ten variables causing the separation of subpopulations (e. g. v2 corresponds to maximal body width). For males, PCA 1 explained 63% of the variations and PCA 2 explained 15%. While Brazilian specimens of *Diplolaimella* sp. were characterized by higher values of body length, maximal body width, pharynx length, spicule length and the ratio of tail length to anal body width, Belgian specimens of *D. dievengatensis* had higher values of ratio of body length to head to gut, the ratio of body length to tail length and the distance between anterior body end and gut as percentage of body length (Fig. 2).

Figure 2. PCA of Brazilian *Diplolaimella* sp. and Belgian *D. dievengatensis* males measurements. Crosses represent Brazilian animals and circles represent Belgian animals. v1 - body length; v2 - maximal body width; v3 - pharynx length; v18 - spicule length; v20 - body length/head to gut; v21 - body length/tail length; v24 - distance between anterior body end and gut as percentage of body length; v26 - head length/head diameter; v27 - pharynx length/head length; v29 - tail length/anal body width

TABLE 1. Males, females and juveniles morphometric data of *Diplolaimella* sp. from Brazil.

	J1				J2				J3				J4				Females				Males			
	mean	min	max	s	mean	min	max	s	mean	min	max	s	mean	min	max	s	mean	min	max	s	mean	min	max	s
L	884.74	784.45	951.91	88.51	930.74	880.45	981.02	71.11	1077.13	1046.31	1122.05	39.79	1264.97	1173.84	1319	79.37	1706.44	1348.68	1890.46	146.63	1343.58	1156.61	1504.00	109.68
m. b. w.	19.15	18.12	20.53	1.24	21.59	21.42	21.75	0.23	27.45	23.96	29.89	3.10	31.92	31.62	32.18	0.28	37.61	29.81	43.97	4.40	25.83	23.89	29.15	1.74
ph. l.	179.45	176	181.89	3.07	178.51	170.78	186.24	10.93	170.83	161.56	175.93	8.04	199.11	178.37	222.46	22.16	234.01	199.30	282.62	23.01	206.56	190.78	218.65	9.79
t. l.	247.02	214.45	269.12	28.80	247.24	230.59	263.88	23.54	329.30	286.85	352.73	36.83	393.21	374.7	408.19	17.02	472.64	398.34	524.93	42.47	335.98	290.65	359.06	23.56
h. d.	7.42	6.68	8.02	0.68	7.65	7.53	7.77	0.17	8.78	8.17	9.25	0.55	9.04	8.57	9.52	0.48	10.33	9.00	12.20	0.93	9.17	7.81	9.78	0.62
h. l.	19.53	17.19	21.76	2.29	20.56	20.29	20.82	0.37	18.00	16.68	18.71	1.15	18.89	17.72	19.93	1.11	21.76	18.28	24.54	2.17	20.71	15.23	23.14	2.36
f. d.	4.72	4.59	4.91	0.17	4.86	4.73	4.98	0.18	5.05	4.61	5.31	0.38	5.14	5.08	5.21	0.07	5.34	4.80	6.07	0.43	5.24	4.71	5.75	0.31
f. b. w.	13.50	12.71	14.09	0.71	12.96	11.64	14.27	1.86	16.02	14.71	16.9	1.16	17.22	16.29	17.89	0.83	18.38	15.28	19.55	1.20	16.34	15.26	18.16	0.93
c. b. w.	19.59	19.34	20.07	0.42	20.29	20.01	20.57	0.40	25.16	21.7	29.07	3.71	27.28	25.67	28.42	1.43	29.75	25.02	32.38	2.71	24.50	22.57	26.23	1.30
a. b. w.	16.51	15.46	17.68	1.12	18.61	17.91	19.3	0.98	21.04	19.8	23.06	1.76	23.56	21.09	26.49	2.73	28.03	22.76	31.17	2.69	24.25	22.14	26.64	1.17
n. r.	102.80	99.86	105.87	3.01	113.08	103.54	122.61	13.48	100.76	99.14	103.23	2.18	107.44	101.32	117.66	8.91	121.57	89.38	154.81	24.79	119.25	85.37	175.47	29.06
g	195.66	193.32	199.79	3.58	193.49	184.99	201.98	12.01	187.27	180.19	191.9	6.23	216.18	195.71	240.99	22.95	256.86	218.13	307.94	24.40	226.07	207.80	240.98	11.15
v or v'	-	-	-	-	-	-	-	-	479.59	413.6	532.13	60.40	612.91	564.26	642.14	42.41	829.99	651.96	909.00	71.54	-	-	-	-
v. a. l. or v'. a. l.	-	-	-	-	-	-	-	-	268.15	217	345.86	68.41	258.85	234.88	280.11	22.74	395.69	298.38	456.53	47.89	-	-	-	-
ph. v. l. or ph. v'. l.	-	-	-	-	-	-	-	-	300.91	233.54	346.48	59.54	406.68	360.7	446	43.04	591.69	443.16	674.34	62.17	-	-	-	-
ph. cl. l.	423.26	386.20	460.32	52.41	-	-	-	-	569.06	539.71	588.08	25.79	665.53	595.58	707.55	60.98	987.37	741.54	1103.21	96.94	792.52	662.70	919.56	80.33
sp. l.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	39.77	36.82	44.79	2.19
gub.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8.74	7.54	9.97	0.88
g. a.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14.29	12.65	17.77	1.83
a	46.17	43.29	48.85		43.14	40.48	45.80		39.50	37.30	43.67		39.63	36.72	41.18		45.78	36.82	54.94		52.22	42.55	58.55	
b	4.52	4.06	4.76		4.81	4.76	4.86		5.75	5.52	5.90		5.89	5.47	6.65		6.66	5.70	7.27		5.94	5.57	6.58	

c	3.59	3.54	3.66	3.77	3.72	3.82	3.29	3.01	3.65	3.22	3.13	3.32	3.61	3.39	3.86	4.00	3.75	4.22
N. R.	11.68	10.79	12.73	12.13	11.76	12.50	9.37	8.90	9.87	8.50	7.78	8.92	7.22	5.26	11.48	8.62	6.49	11.67
V ou V'	-	-	-	-	-	-	44.44	39.53	47.42	48.44	48.07	48.68	48.68	46.16	51.54	-	-	-
G	22.25	20.99	24.64	20.80	20.59	21.01	17.40	16.95	18.13	17.14	17.10	17.22	15.08	13.75	17.56	16.88	15.19	17.97
f. b. w./f. d.	2.87	2.59	3.03	2.68	2.34	3.02	3.17	3.10	3.23	3.35	3.21	3.43	3.46	2.92	3.86	3.13	2.82	3.44
h. l./h. d.	2.63	2.45	2.88	2.69	2.68	2.69	2.05	2.02	2.09	2.09	2.07	2.11	2.12	1.81	2.47	2.27	1.73	2.96
ph. l./h. l.	9.29	8.09	10.58	8.68	8.42	8.95	9.53	8.63	10.49	10.54	9.37	11.16	10.81	9.13	12.34	10.07	9.23	12.62
v. a. l./a. b. w.	-	-	-	-	-	-	12.88	10.48	17.47	11.05	10.17	12.40	14.21	11.92	17.05	-	-	-
t. l./a. b. w. = c'	14.96	13.87	16.43	13.27	12.87	13.67	15.66	14.49	17.40	16.85	14.98	19.35	17.00	13.72	20.00	13.88	11.68	16.04
sp. l./a. b. w.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.64	1.50	1.81
t. l./v. a. l.	-	-	-	-	-	-	1.30	0.83	1.63	1.52	1.42	1.60	1.20	1.10	1.34	-	-	-

Abbreviations (adapted from Jacobs et al., 1990): a: ratio of body length to maximal body diameter (L/m.b.w); a. b. w.: anal body width; b: ratio of body length to distance between anterior body end and gut (L/g); c: ratio of body length to tail length (L/t); c. b. w.: cardial body width; f. b. w.: average of the body width at the center of both amphidial fovea; f. d.: outer fovea aperture diameter measured parallel to central body axis; g: distance between anterior body end and gut; G: distance between anterior body end and gut as percentage of body length (%); gub: gubernaculum length; g. a.: gubernaculum apophyses length; h. d.: head diameter = body width at cephalic setae measured perpendicular to central body axis; h. l.: head length = distance between anterior body end and anterior margin of both amphidial fovea, measured along central body axis; JI-J4: juvenile stages 1 to 4; L: body length; m. b. w.: maximal body diameter; n. r.: distance between anterior body end and nerve ring; N. R.: distance between anterior body end and nerve ring as percentage of body length (%); ph. c1. l.: distance between pharynx-cardia junction and cloacal orifice; ph. l.: pharynx length; ph. v. l.: distance between pharynx-cardia junction and vulva; ph. v'. l.: distance between pharynx-cardia junction and posterior margin of genital primordium; sp. l.: spicule length measured along the arc; t. l.: tail length; v: distance between anterior body end and vulva; v': distance between anterior body end and posterior margin of genital primordium; V: distance between anterior body end and vulva as percentage of body length (%); V': distance between anterior body end and posterior margin of genital primordium as percentage of body length (%); v. a. l.: distance between vulva and anus; v'. a. l.: distance between posterior margin of genital primordium and anus.

While for females, the first three components explained 79% of the variations (PCA 1 = 34%, PCA 2 = 27% and PCA 3 = 18%). Brazilian specimens were characterized by higher values of body length, a, c, and the ratio of tail length to distance between vulva and anus, and Belgian specimens had higher distance between anterior body end and gut, b, the distance between anterior body end and gut as percentage of body length and the ratio of pharynx length to head length (Fig. 3).

Figure 3. PCA of Brazilian *Diplolaimella* sp. and Belgian *D. dievengatensis* females measurements. Crosses represent Brazilian animals and dots represent Belgian animals. v1- body length; v13 - distance between anterior body end and gut; v19 - a; v20 - b; v21 - c; v24 - distance between anterior body end and gut as percentage of body length; v27 - pharynx length/head length; v28 - distance between vulva and anus/anal body width; v29 - tail length/anal body width; v32 - tail length/distance between vulva and anus.

Lastly, for juveniles PCA 1 and PCA 2 explained 82% of the variations (62% and 20% respectively). Brazilian J1 and J2 had higher values of body length, maximal body width, head length, anal body width, a, b and the ratio of pharynx length to head length. The distance between anterior body end and posterior margin of genital primordium and the distance of vulva to anus/anal body width were not measured in these animals because genital structures were inconspicuous. Brazilian J3 and J4 had higher values of body length, maximal body width, head length, anal body width and the distance between anterior body end and posterior margin of genital primordium, while the ratio of head

length to head diameter and the distance of vulva to anus/anal body width were higher in Belgian J3 and J4 (Fig. 4).

Figure 4. PCA of Brazilian *Diplolaimella* sp. and Belgian *D. dievengatensis* juveniles measurements. Unfilled circles represent Brazilian J1 and J2, and unfilled triangles represent Brazilian J3 and J4; filled circles represent Belgian J1 and J2, and filled triangles represent Belgian J3 and J4. v1 - body length; v2 - maximal body width; v6 - head length; v10 - anal body width; v14 - distance between anterior body end and posterior margin of genital primordium; v19 - a; v20 - b; v26 - head length/head diameter; v27 - pharynx length/head length; v28 - distance between vulva and anus/anal body width.

In general, males, females and juveniles from Brazil and Belgian species are separated by measures of body length, maximal body width, head length, pharynx length, the distance between anterior body end and gut, the distance between vulva and anus, spicule length and tail length, Brazilian animals being isometrically larger than Belgian animals.

Life cycle parameters

The eggs started to hatch on the third day after they have been laid, when the hatching rate was of 60%. In the fourth day, the rate reached 86% and thereafter no more hatching was observed. Thereby the hatching time was estimated as $3.3 \text{ days} \pm 0.5$. The egg

size had $23.4\mu\text{m} (\pm 2.22) \times 46.6\mu\text{m} (\pm 5.57)$. Unhatched eggs remained in the gastrulation phase, and did not develop into a juvenile nematode. Somatic length-based growth was followed throughout twenty-eight days. The newly hatched juveniles measured $244.93 \pm 27.81\mu\text{m}$ and had an estimated biomass of $0.013 \pm 0.003\mu\text{g}$ (Fig.5 and 6). Both body length and biomass increased linearly in juveniles and stabilized after sexual differentiation (Fig.5 and 6). The sexual differentiation started in the sixth day, when females were observed measuring $763.21 \pm 115.65\mu\text{m}$ and weighing $0.137 \pm 0.05\mu\text{g}$. Males were only observed from the seventh day on measuring $821.51 \pm 96.26\mu\text{m}$ correspondents to $0.173 \pm 0.03\mu\text{g}$, when the reproductive period started. On the same day, gravid females were observed in the plate measuring $1002.33 \pm 50.03\mu\text{m}$ correspondent to $0.200 \pm 0.03\mu\text{g}$. These measures seem to be the minimum body length and biomass necessary for females to reproduce. The females grow until the fourteenth day, when the body length remained in $1133.01 \pm 15.22\mu\text{m}$ and biomass $0.425 \pm 0.07\mu\text{g}$ up to the end of experiment. For the males, the growth was until the sixteenth day thereafter the body length remained in $1017.05 \pm 9.92\mu\text{m}$ and biomass reached $0.323 \pm 0.08\mu\text{g}$. Calculation of the intrinsic rate of natural increase (r_m) produced a value of 0.46 for *Diplolaimella dievengatensis*, corresponding to a population doubling time of 1.48 days. The capacity for increase (r_c) was 0.43 and the values of the three alternative measures of mean generation time, T_0 , T_1 , and T were 6.90, 7.17 and 5.43, respectively (Table 2).

Figure 5. Growth of *Diplolaimella dievangatensis* based in body length (μm) from juveniles up to females and males adults. Dotted lines: 95% CIs for the regressions. The regression lines for the juveniles growth shown ($\pm 95\%$ CIs in parentheses) are: body length = $81.88 (\pm 2.16) \times \text{day} + 128.57 (\pm 8.48)$ ($r^2 = 0.77$, $p < 0.001$). Red circles filled black indicate measures of the first pregnant females observed. Each point represents one observation.

Figure 6. Individual biomass (μg) of *Diplolaimella dievangatensis* from juveniles up to females and males adults. The regression lines for the juveniles biomass shown ($\pm 95\%$ CIs in parentheses) are: biomass = $0.016 (\pm 0.0007) \times \text{day} + 0.0106 (\pm 0.0025)$ ($r^2 = 0.60$, $p < 0.001$). Red circles filled black indicate measures of the first pregnant females observed. Each point represents one observation. Green line with stars indicate measures of juveniles; red line with red circles filled and blue line with blue circles filled indicate measures of biomass females and males from European *Diplolaimella dievangatensis*, respectively at 20°C (Vranken et al, 1987).

Table 2. Life cycle demographic parameters of *Diplolaimella dievengatensis* from Brazil, at 25°C and 20 salinity.

Hatching time in days (H_t)	3.3 ± 0.5
Rate of natural increase (r_m)	0.41
Capacity for increase (r_c)	0.36
Population doubling time in days (PDT)	1.48
Mean age in days for reproduction of a cohort of females (T_0)	6.60
Period of time necessary for population grow (T_I)	7.17
Age in days of the mothers of a set of newborns in a population (T)	10.3

Molecular analyses

We obtained a sequence with 1713 pb. The alignment showed only ten nucleotides differences between our sequence and a sequence of *Diplolaimella dievengatensis*, what corresponds to a similarity percentage of 99.4%.

DISCUSSION

Measurements

Despite the Brazilian nematode species had eight characters isometrically larger than Belgian animals, the presence of the set of diagnostic features (Jacobs *et al.*, 1990; Fonseca & Decraemer, 2008) determined that both species were morphologically similar. According to Venekey *et al* (2010) the only species of this genus previously recorded in Brazil is *Diplolaimella chitwoodi* (Gerlach, 1957), which was found in the Cananea estuary. In this sense, this is the first record of *Diplolaimella dievengatensis* for Brazil. This species was originally described in “Dievengat”, a brackish water pond near the city of Knokke, northwestern Belgium coast (Jacobs *et al*, 1990) and it has been defined from its type locality. *Diploalimella dievengatensis* species inhabits typical marine or brackish-water systems and composes an “Aufwuchs community”, which consists in a nematode association with macrophytes, cyanophytes and decaying materials drifted ashore (T. Moens & Vincx, 1998; Da Rocha *et al.*, 2006). It is primarily bacterivore, classified as nonselective deposit feeder (Wieser's, 1953) and easily cultivated in agar (Vranken *et al.*, 1984; Vranken, 1987). Despite the high similarity, the Brazilian and Belgian specimens differed in the size of the animals, with the Brazilian being larger than Belgian individuals.

It is important to note that only nematodes used to morphometric analyses were larger, whereas those used on life cycle experiment had similar adult body size. This discrepancy can be due to the different methods to prepare specimens (viz. anesthesia, fixation and/or mounting causing contractions and deformations) the equipment used in the biometry, or intraspecific variation (Oliveira et al., 2010; Oliveira *et al.*, 2012). Nematodes in cultures are small population, subject to inbreeding and demographic stochastic, although this is often avoided by nematode multi-transfer to new plates.

Molecular analyses

A difference of less than 1% between sequences of the molecular region analyzed is considered within the intraspecific variation of marine nematodes (Bhadury *et al.*, 2006). Therefore, the molecular data indicated that our specimen is *Diplolaimella dievengatensis* or, at least, belongs to the same species complex. Although cryptic diversity is frequently described to marine meiofauna, it was recently demonstrated that this is not always the rule (Kieneke *et al.*, 2012; Oliveira *et al.*, 2017). However, to confirm that *D. dievengatensis* and the *Diplolaimella* used in present study do not form a group of cryptic species, it is necessary to analyze molecular markers that have more variability within populations of marine nematode species. Free-living marine nematodes have a limited dispersal capability mainly due to absence of planktonic or pelagic larvae and their small body size, which restricts active dispersal over large distances. Despite of that, many species apparently have a broadly distribution creating a ‘meiofauna paradox’ (Giere, 2009). Since our data support the hypothesis of an amphi-oceanic distribution of *D. dievengatensis*, this strengthened the paradox. Such long distance dispersal may be human mediated, through ballast water for example (Radziejewska *et al.*, 2006), or may be related to current-driven sediment transport (Boeckner *et al.*, 2009).

Life cycle and population growth parameters

Species of the Monhysteridae family are highly successful laboratory cultures and develop within a wide salinity and temperature range (Moens & Vincx, 1998). In this study, the salinity and temperature used were similar to the field condition, where this species was collected. The hatching rate of *Diplolaimella dievengatensis* was 86%. This is a high hatching success and combined with others parameters, makes this species suitable for use

in laboratory assays or ecotoxicological screening tests (Palma *et al.*, 2009). High hatching rate (96%) was also found for other monhysterid species, *Monhystera disjuncta* (Vranken & Heip, 1986). The egg size of *D. dievengatensis* ($23.4 (\pm 2.22) \times 46.6 \mu\text{m} (\pm 5.57)$) were considerably larger than eggs of another species of the family Monhysteridae, *Monhystera parelegantula* ($20 \times 26 \mu\text{m}$, Vranken *et al.* (1981). Egg dimensions may change within of family or genus and differences in demographic strategies can also influence size as well as the speed of egg production (Zullini & Pagani, 1989). The hatching time of the Brazilian species (3.3 ± 0.5 days) was similar to the European *D. dievengatensis* (3.0 ± 0.9 days) (Vranken, 1987), in the same abiotic conditions. For the European species, salinity and mainly temperature had high influence in the hatching time. It was observed that at lower temperature (i.e. 15°C) the embryonic development time doubled whereas at higher temperature (i.e. 30°C), it was reduced to a half.

The somatic length-based growth of Brazilian *D. dievengatensis* juveniles suggested a pattern of continuous and linear growth, which differs from the exponential growth pattern observed in juveniles of the European species (Vranken, 1987). Nematodes typically increase in size throughout the inter-moult periods but, whether the growth pattern of juveniles is a continuous or a discontinuous process is still undefined. In some species a slight slowing in the rate of somatic growth, called “lethargus” phases, has been detected immediately before or during moults (Lee, 2002). However, patterns of continuous growth have been reported in most studies (Muschiol & Traunspurger, 2007; Ehlers *et al.*, 2013). Nonetheless, the apparent absence of saltational growth can be also explained by methodological limitations, such as lack of synchrony in the developmental phases of individuals or a longer time resolution of measurements, which may suggest continuous pattern. Other observers have implied that the impact of moulting on the growth curve is so small as to be unmeasurable (Wilson, 1976).

The Brazilian *D. dievengatensis* had similar size to the Belgian population as the body length (Jacobs *et al.*, 1990). The cultivated females measured $1133.01 \pm 15.22 \mu\text{m}$ and the males, $1017.05 \pm 9.92 \mu\text{m}$, whereas individuals from Belgium measured $1126.6 \pm 99.9 \mu\text{m}$ and $1039.4 \pm 62 \mu\text{m}$ respectively. Cultivated species of monhysterids do not reach over $2000 \mu\text{m}$ in body length (Vranken *et al.*, 1981). Furthermore, like in other species of this family, females of *D. dievengatensis* were larger than males (Vranken *et al.*, 1988). Numerous species of nematodes starts to lay their eggs considerably earlier before reaching

maximum size (e.g. *Aphelenchus avenae*, which start egg-laying at 51.5% of final size (Soyza, 1973). On the other hand, *D. dievengatensis* starts to lay their eggs at about 90% of final size. The differences in maturation time relative to the final body size may be related to distinct life history strategies (Nylin & Gotthard, 1998).

Similarly to the body length, the individual biomass of the Brazilian *D. dievengatensis* resembled that of the Belgian population, mainly in the juvenile phase. Yet females also had higher biomass than males, keeping a difference of about 0.3 μg (Fig. 6). However, it is important to note that Belgian specimens were cultivated at 20°C, while our specimens were cultivated at 25°C (Fig. 6). It can be expected that at higher temperature the body size after the last moult to be smaller than at lower temperature. Higher temperature increase anabolic oxygen demand, decreasing oxygen solubility. The supply oxygen constrain affect aerobic scope harming the individual performance, therefore, this unbalance may result in smaller final body sizes (Baudron *et al.*, 2014). For example, in the nematode *Stottinema lindsayae*, at 15°C the final body weight was one third of those at 10°C (Overhoff *et al.*, 1993).

The intrinsic rate of natural increase (r_m) for Brazilian *D. dievengatensis* ($r_m = 0.41$) was higher than that of the European population ($r_m = 0.348$), under the same salinity and temperature (Vranken *et al.*, 1984). Values of r_m above 0.2 day⁻¹ are considered high for nematodes, characterizing opportunistic species (Warwick, 1981; Banse, 1982). The population doubling time was 1.48 days, which evidence a highly productive species, such observed in other bacterial-feeding species (Muschiol & Traunspurger, 2007). In short the intrinsic rate of increase is influenced by survival, growth, and fertility rates, which define the life history characteristics of a species or population (Heppell *et al.*, 2000). External factors may affect such rates, as for example food availability which is directly related to fertility rates (Heip *et al.*, 1985). Few studies have investigated the role of food quality on r_m , however, *Diplolaimella chitwood* had an $r_m = 0.302$ when feeding on detritus from *Spartina alterniflora* and lower values under other food regimes (Findlay, 1982). Other feature that influences r_m is the generation time, which is negatively correlated with r_m (Pianka, 1970). Thus, species with shorter generation time have higher potential to rapidly increase their population (Soulhwood 1976).

The generation time of free-living nematodes may vary between a few days (e.g. rhabditids) to months (e.g. oncholaimids) (Tietjen & Lee, 1977a; Ferris & Ferris, 1979;

Heip et al, 1985). Estimates of generation time are useful parameters for predicting aspects of in situ population dynamics, biomass, and productivity (Vranken & Heip, 1986). Nevertheless, the great variety of definitions for generation times estimates among laboratory studies with free living marine nematodes make comparisons very difficult. Among these definitions, we used the T (i.e. the mean age of mothers of newborn individuals in a population with stable age distribution), which is preferred over other measures (Charlesworth, 1994). All other definitions can also be considered approximations to the mean generation time (Heip *et al.*, 1985). Although Vranken (1987) have used the $T_{\min}$ (time interval between the same life stages) for estimating generation time of the Belgium *D. dievengatensis*, this parameter is adequate only for semelparous individuals and organisms reproducing by fission (King, 1982). For iteroparous organisms, $T_{\min}$ measures only development time, since the reproduction is continuum in most free-living nematodes. The $T_{\min}$ of the Belgium *D. dievengatensis* was 14 days (Vranken, 1987), for *Diplolaimella ocellata* (24°C and 15 salinity) was 11.5 (Hopper *et al.*, 1973b), and the T of the Brazilian specimens of *D. dievengatensis* was 10.3 days, which is comparable to that obtained for other marine monhysterids. This value allows more than 15 generations per year, what can be considered a high number of generations compared to other nematodes.

In general, the studied life cycle parameters of the *D. dievengatensis* from Brazil agreed well with those obtained from cultures of individuals isolated in Belgium (Vranken & Heip, 1986; Vranken, 1987). It demonstrates that populations exposed to distinct environmental conditions exhibited similar life cycle patterns under laboratory cultures. Although Vranken (1987) have not shown the parameters used to estimate the r_m , the higher r_m of *D. dievengatensis* from Brazil can be explained by lower generation time and, at lesser extent, by the higher survival of females in the population. Further comparison was not possible due to methodological constraints, since no detailed information were presented in the previous studies (e.g. food quality and concentration). Laboratory cultures provide detailed and highly useful information on nematode life cycles, which can be used to predict field conditions. Because of the similarities in life cycle parameters between the different populations, the viability of cultures and wide distribution ranges, we considered that *D. dievengatensis* is a useful model species for experimental studies.

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CAPÍTULO II

Response of marine nematodes to stress depends on species life history strategy

Response of marine nematodes to stress depends on species life history strategy

ABSTRACT

According to species life-history strategies, species can be ranked along a “fast-slow continuum” where “slow” species (i.e., lower fecundity, long lived and late maturing) are expected to be more tolerant to stress conditions at the individual level whereas “fast” species (i.e., higher fecundity, short lived and early maturing) to be less tolerant. In this study, we have compared the response of different life cycle parameters of two free-living marine nematodes with different life-history strategies, to the exposure to sublethal concentrations of sodium dodecyl sulfate (SDS) surfactant. We have analyzed the effects of SDS exposure on the growth rate, reproduction rate and survivorship of the faster species *Litoditis marina*, and of the slower species *Diplolaimella dievengatensis*. Exposure to SDS significantly affected the growth rate and reproduction rate of both species. However, whereas growth and reproduction rates of the slower species were significantly enhanced at low and intermediate concentrations of SDS (0.001% and 0.003%), for the fast species both parameters were significantly reduced by exposure to all SDS concentrations tested (0.001%, 0.003% and 0.006%). SDS exposure did not affect the survival of adult nematodes of the slow species. Survival of adult individuals of the fast species, on the other hand, was significantly affected by SDS and this effect was dependent on adult gender. Whereas survival of adult females did not differ between the control and SDS treatments, survivorship of males exposed to 0.006% SDS was significantly reduced compared to the control. Although both species are relatively close along the fast-slow continuum, they responded distinctly to the stress caused by sublethal concentrations of SDS. *L. marina* had a reduced body growth and reproduction rates under SDS exposition, which meets the trend of faster species in allocating fewer investments on defenses against physiological injuries and on their own somatic maintenance. On the other hand, the slower *D. dievengatensis* had higher survivorship rates and enhanced reproduction and growth rates under stress. We propose that the apparent lower resistance of this faster species under stress at the individual level is balanced by their higher reproductive rates, conferring higher resilience to these species at the population level. Identifying and understanding differential effects of stress in the context of life-history theory is an important aspect to enhance our understanding about the ecological threats posed by anthropogenic activities.

KEY WORDS

Free-living marine nematodes, life cycle history, fast-slow continuum, SDS

INTRODUCTION

Under stress, organisms may have to increase energy expenditure to withstand and therefore deal with additional costs in terms of energy maintenance (Calow & Forbes, 1998). Such costs may decrease the energy available for other functional outputs like growth and reproduction, which can ultimately lead to reduced population growth and survival (Calow & Sibly, 1990). For this reason, quantification of individual life-cycle traits has become a useful tool to assess the risk of anthropogenic pollutants on ecosystems (Kammenga et al., 1996; Nørhøve et al., 2012; Jager et al., 2014). Individual growth, reproduction and survival have been the most commonly used parameters (Hansen et al., 1999; Roh et al., 2007). In most cases, however, these parameters were assessed in isolation (Jager et al., 2004) and knowledge about the interplay between them is to some extent limited. Yet, understanding how stressors affect the life-history strategies across species provide useful insights to further understand the effects on population dynamics and, ultimately, on ecosystems (Quetglas et al., 2016).

The current life-history theory proposes that species can be ranked along a “fast-slow continuum” concept (Zera & Harshman, 2001; Ricklefs & Wikelski, 2002), which origin was based on the r/K-selection (Reznick et al., 2002; Reynolds., 2003). In this concept, species have different energy investments in life cycle traits which result from a physiological trade-off between growth, reproduction and survival (Calow and Sibly, 1990). For instance, fast species (similar to r-strategists) invest mostly on reproduction (e.g. increased mating ability, fecundity and fertility), at the cost of reduced energetic investments on somatic growth and maintenance (Kirkwood & Holliday, 1979; (Harshman & Zera, 2007; Garratt et al., 2013). Slow species (similar to K-strategists), on the other hand, spend more energy on individual growth and maintenance and less on reproduction. As a result, fast species are characterized by small body size, short lifespan, early reproduction, high fecundity, small offspring size and low inherent survivorship (Jeschke & Kokko, 2009). Slow species, conversely, have opposite characteristics.

Species response to stress will likely vary according to species life-history strategies. In the case of fast species, for instance, the reduced energetic investment in somatic maintenance makes them, at the individual level, less resistant to stress (Hoffmann & Parsons, 1989). At the population level, however, the high population growth and

turnover rates (resultant from the large investment on reproduction) confer them higher resilience on the ecosystem (Begon et al, 2005). Species with slow life histories, on the other hand, are endowed with efficient anticipatory mechanisms and are therefore able to recruit energy resources to optimize their survival performances under stress (Ponge, 2013), in detriment of fecundity (Palacios et al., 2012; Aktipis et al., 2013). Therefore, these species are expected to tolerate stress conditions at the individual level (high resistance), but have limited recovering capacity at the population level (low resilience).

In this study, we compare the responses of life cycle parameters (growth, reproduction and survivorship) of two free-living marine nematodes with different life-history strategies, to the stress posed by the exposure to sub-lethal concentrations of SDS surfactant. Free-living nematodes represent one of the most abundant and species-rich metazoan groups in marine and freshwater sediments and soils (Giere 2009) and have a remarkable diversity of life-history strategies, comprising the “fast-slow continuum”, with species both tolerant and sensitive to pollutants (Bongers, 1990). We have chosen as “fast species” the cultivable species *Litoditis marina* (Bastian, 1865) Sudhaus, 2011, whose life cycle traits include very short generation time and high fecundity (Table 1). As “slow species”, we have chosen *Diplolaimella dievengatensis* (Jacobs et al., 1990) that has a relatively longer generation time and lower fecundity (Table 1). Despite females of both species have similar body length at egg production; *L. marina* continues to grow after maturation whereas *D. dievengatensis* does not (Table 1). The earlier maturation and shorter generation time of *L. marina* indicate lower energy investment in somatic growth compared to *D. dievengatensis*.

The SDS detergent, herein utilized as the chemical stressor, is often used in ecotoxicological tests as a reference substance (Jorge & Moreira, 2005; Mariani et al., 2006; Faimali et al., 2014). It has received special attention in the last decades since it is used as a residual of anionic tensoactive agent for household, agricultural and industrial purposes (Singh et al., 2007) and has proved to have detrimental effects on the biota (Messina et al., 2014). Specifically, we test the hypothesis that the effect of SDS on individual life-cycle traits of *L. marina* and *D. dievengatensis* will be dependent on the species life-history strategy. Since slower species are expected to prioritize self-maintenance and survival, at the expense of reproduction, we expected that individual

growth and survivorship rates of *D. dievengatensis* would be less impacted by SDS exposition when compared to *L. marina*, whereas, reproduction, would be more affected.

Table 1. Life-history characteristic of *D. dievengatensis* and *L. marina*.

Life-history characteristic	<i>D. dievengatensis</i>	<i>L. marina</i>	Source
Minimum generation time	7.8 days F 8.0 days M	3.24 ($\pm$ 0.4) days	Vranken, 1987; Moens & Vincx, 2000
Reproductive period	24-41 days	6-8 days	Vranken, 1987; Tietjen et al, 1970
Daily egg production	12.2 ($\pm$ 1.7)	18.27 ($\pm$ 5.0)	Vranken, 1987; Moens & Vincx, 2000
Embryonic development/ egg hatching	3.0 ($\pm$ 0.9)	0.5 - 1 day	Vranken, 1987; Vranken & Heip, 1983
Survivorship	$\leq$ 80 days F $\leq$ 240 days M	60% females die after copulation	Vranken, 1987; Tietjen et al, 1970
Body length total	1150 μ m ($\pm$ 100) F 1050 μ m ($\pm$ 50) M	1400 μ m F 1120 μ m M	Oliveira et al, <i>in prep</i> ; Tietjen et al, 1970
Body length(eggs production)	990 μ m ($\pm$ 90)	935-960 μ m	Oliveira et al, <i>in prep</i> ; Tietjen et al, 1970

The life history characteristics are based in experiment performed to 20-25 of salinity, 20° and 25° of temperature to *D. dievengatensis* and *L. marina*, respectively.

MATERIALS AND METHODS

Cultivation of nematodes

Individuals of *Diplolaimella dievegatensis* were obtained from the Guaratuba Estuary, Brazil and *Litotidis marina* from the Westerschelde Estuary, Netherlands. The latter was obtained from the Marine Biology Section of Ghent University.

Both species are bacterial-feeding nematodes considered as colonizers *sensu* Bongers (1990). The *L. marina* culture used in the present experiments belongs to the PmI lineage which is the most abundant and widespread lineage in the Westerschelde Estuary (Derycke et al., 2006). The nematode *D. dievegatensis* was for the first time recorded in Brazil and was isolated in a monospecific culture. This culture has been kept in the laboratory under controlled conditions since 2012. The detailed description of methods employed to artificial culture systems is given in Moens & Vincx (1998). Culture stocks of both species were maintained in the dark, on bacto-nutrient agar media prepared with artificial seawater (ASW; Dietrich and Kalle, 1957) under salinities and temperatures of 20 and 25°C for *D. dievegatensis* and of 25 and 20°C for *L. marina*, respectively. This optimum abiotic condition was defined in previous studies (Moens and Vincx, 1998; Tietjen et al., 1970). Only nematodes from cultures in exponential growth phase were used for the experiments.

Experimental conditions and media

All experiments were conducted in polystyrene Petri dishes (5 cm of diameter) filled with bacto-agar medium (DIFCO) at volumes of 3 ml and concentrations of 0.6 % for *D. dievegatensis* and of 4 ml and 1% for *L. marina*. The distinct volumes (*i.e.* the thickness of the agar layer) and bacto-agar concentrations were used to suit to each species behavior. Lower agar concentrations facilitate the penetration and movement of *D. dievegatensis* and the thinner layer of agar is suitable to prevent this species from getting trapped in the bottom of the dish (Moens and Vincx, 1998). The pH of the medium was buffered at 7.5–8 with Tris – HCl (Tris (hydroxymethyl)aminomethane-HCl) at a final concentration of 5 mM. Frozen-and-thawed suspensions of the bacterial strain *Escherichia coli* ATCC 11229 were used as food source at densities of 5×10^8 and 3×10^{10} cells ml⁻¹, which are the optimal food concentrations for *D. dievegatensis* (Moens et al., 1999) and *L. marina* (dos Santos et al., 2008), respectively. Cholesterol (125 µLl⁻¹) was added as a source of sterols, because nematodes are incapable of synthesizing sterols when their only food source is bacteria (Vanfleteren, 1980).

The SDS treatment concentrations were 0.001%, 0.003% and 0.006% [w/v], and followed the study of Harada et al (2007) which observed sublethal effects on growth and reproduction of *Caenorhabditis elegans* at these concentrations. The SDS (Sigma –

Aldrich, 98.5% purity) was dissolved in ASW and autoclaved to produce a stock concentration of 1.5 mg.l^{-1} , which in turn was serially diluted. Petri dishes containing the bacto-agar medium received each 120 μl of the stock solution, which was immediately spread to reach the SDS final concentration. Plates were placed on a clean bench at room temperature for 10 min and then 50 μL of an *E.coli* suspension was added to each plate. Food was provided only at the beginning of the experiment.

Hatching success and growth rate

The hatching experiment was performed only with *D. dievengatensis*, because eggs of *L. marina* can undergo intrauterine development (Moens & Vincx, 2000), which may prevent possible effects of exposition to SDS. Eggs of *D. dievengatensis* were obtained from egg-laying females, which were kept over 24 hours in agar plates under contaminated (0.001%, 0.003% and 0.006%) and control agar medium plates, for synchronization. Fifteen eggs from each treatment were transferred to fresh plates with the same SDS concentration from the original plates. Five replicate plates were set to each treatment. The hatching success was determined as the percentage of total eggs that has hatched at day four, since egg-laying in *D. dievengatensis* occurs within this interval (Vranken, 1987). Plates were observed under an inverted microscope (Nikon Eclipse TE 300).

Growth rate was obtained for both species. Juveniles of *D. dievengatensis* from eggs hatched in the previous experiment were used to estimate the effects of SDS on J1 body length, total body length and individual growth rate. These last two parameters were estimated through daily body length determinations for a total of six days. Measurements were taken from all individuals in the plates. Since hatching was not the same in all plates and the number of individuals decreased throughout the experiment, the number of measures per plate, per day, was not constant. For *L. marina*, juveniles were obtained from egg-laying females maintained over 24 hours on contaminated and control plates. Ten juveniles were transferred to each fresh plate with the same SDS concentrations from the original plate. Two replicates were set per treatment and seven to nine nematodes per plate were observed daily for a total of six days. The nematodes were photographed under inverted microscope equipped with a Nikon D300 camera and analyzed using the image analysis software *ImageJ* (<http://rsbweb.nih.gov/ij/>).

Reproductive rate and Survival

Newly matured males (n=6) and females (n=10) were inoculated per plate for the same treatments applied in the hatching and growth rate experiments. The reproductive rate (*Rr slope*) was determined as the number of juveniles and eggs at the first generation (F1) divided by the numbers of females (F0) throughout the first eight days, after which sexual maturity was reached. The total fecundity at the end of experiment was also observed. To avoid overlap between parental and progeny generations, males and females of the F0 generation were transferred to new test plates for egg laying every 36 hours for *L. marina*. Since females of *D. dievengatensis* proved to be very sensitive to plate transfer, the total numbers of eggs and juveniles were counted daily until the maturation of the F1 generation. For each SDS concentration and control, four and five replicate plates were prepared for *D. dievengatensis* and *L. marina*, respectively. Survival was measured in males and females from F0, which had been exposed to SDS since the beginning of life.

Data Analyses

All statistical analyses were performed in R version 3.1.1 (R Core Team, 2014). A specific statistical model was used to each parameter according to the nature of the data set (Table 2). Effects of SDS on growth were analyzed by comparing the individual growth rate (*slope Rr*) of nematodes over time between treatments, as well as the J1 body length and the body length at the end of the experiment. For that, we have used generalized mixed-effects models (*lmer*) of the *lme4* package (Pinheiro et al., 2014). Since some individuals died or escaped from the plate during the experiment, our experimental design was unbalanced. A mixed-effects model for the growth analysis of unbalanced experiments offers advantageous flexibility by handling with missing data, because it allows all observational units to contribute with information to the analysis in a unified framework (Pinheiro & Bates, 2000). In addition, by allowing regression coefficients to vary randomly across individuals (random effect), mixed-effects models are capable of dealing with observational heterogeneity and the non-independent observations (Sackman et al., 2008). The body length measurements were transformed to logarithmic scale and the normality of the residuals was graphically explored. The *lmer* model was applied using body length as response variable and the interaction between treatment (control, SDS) and Days (0-6 days) as predictors. Treatment was considered as categorical and days as

continuous variables. This same design was used for *Rr* (Reproductive rate) and survivorship. The best-fit model was selected by AICc smaller than two (Akaike's Information Criterion) (Burnham and Anderson, 2002) and then re-fitted with restricted maximum likelihood (REML). To verify if the Treatment factor had a significant effect on growth, a null model without this factor was constructed and compared by likelihood ratio test with the best fit-model. When significant differences between models were detected, a *post-hoc* multiple pairwise comparisons using the contrast matrix with the *glht* function (package *multcomp*) with Bonferroni corrections were performed. The SDS effects on the slope of reproductive rate (interaction between SDS and Days - *Rr*) and on total fecundity at the end of the experiment were tested using generalized least squares (GLS) with REML (Zuur et al., 2009). Exploratory analyses revealed heterogeneity in the variance of the residuals. For that, we fitted the data specifying the variance structure using the *nlme* R package (Pinheiro et al., 2011). The lowest AICc was used to select the most appropriate variance structure (Zuur et al., 2009). For *D. dievengatensis*, the best model used a combined variance structure for Treatment and for Days. The variance of treatment was handled with “varIdent” which assumes a different variances per stratum, while the variance of Days was treated with “varExp” which considers the exponential of the variance covariate. For *L. marina*, the best model was based uniquely on constant power of the variance covariate function. To the analysis of *Rr*, the temporal correlation function was used as compound symmetry correlation structure [CorcompSym (form= ~ Days)] for *D. dienvengatensis* and an auto-regressive moving average [ARMA(1,0)] for *L.marina* models. The likelihood ratio test was applied to the best models with the chosen autocorrelation and variance structure (Zuur et al, 2009). If significant differences in the interaction term Treatment x Days were detected, a *post-hoc* multiple pairwise comparisons was conducted as mentioned above. Finally, the survivorship and hatching success parameters were analyzed by GLM (generalized linear model) with binomial distribution for proportion data. Accumulated data from the last experimental day was used as response variable for both experiments. For survivorship, the predictor Gender was added to the models, to test if the effect of SDS was dependent on the gender. The significance of the interaction between factors was done by the likelihood ratio test using the Chi-square function as an approximation. If significant effects were detected, post-hoc multiple pairwise comparisons were used.

Table 2. Overview of statistical analysis for the different parameters of experiments.

<i>Parameter</i>	<i>Model</i>	<i>Distribution</i>	<i>Predictors</i>
Hatching	GLM	Binomial	Treatment
Growth rate	<i>lmer</i>	Normal	Treatment x Days
J1 body length			Treatment
Total body length			Treatment
Rr slope	GLS	Normal	Treatment x Days
Total fecundity			Treatment
Survivorship	GLM	Binomial	Treatment + Gender

RESULTS

Hatching success and Growth rate

Hatching success of *D. dievengatensis* varied from 59% (± 9.6) to 76% (± 3.7) and did not differ between treatments ($\chi^2 = 1.28$, $p = 0.732$; Fig. 1). The best-ranked growth rate model for *D. dievengatensis* was $AICc = -1811.0$, $dAICc = 0$, $df = 12$, $weight = 0.75$ ($Body_{length}(\log_{10} [+1]) \sim \text{Treat.} * \text{Day} + (\text{Day} | \text{Plate})$) and of *L. marina* was $AICc = 5282.4$, $dAICc = 0$, $df = 11$, $weight = 1.0$ ($Body_{length}(\log_{10} [+1]) \sim \text{Treat.} * \text{Day} + (\text{Day} | \text{Plate})$). Eggs of both *D. dievengatensis* ($\chi^2 = 3.91$ (3), $p = 0.271$) and *L. marina* ($\chi^2 = 5.73$ (3), $p = 0.125$) hatched into first-stage juveniles (J1) with similar body length for all treatments (Table 3.) SDS significantly affected the growth rate of *D. dievengatensis* ($\chi^2 = 194.04$ (7), $p < 0.001$) and *L. marina* ($\chi^2 = 92.76$ (7), $p < 0.001$) (Fig. 2 a, b). Growth rate of *D. dievengatensis* at 0.003% was significantly higher than at all other treatments (Table 4), whereas at 0.001% and 0.006%, it was similar to the control (Table 4). For *L. marina*, growth rates were significantly lower at all SDS treatments when compared to the control (Table 4). SDS effects on growth rates were reflected on total body length of both species at the end of the experiment (Table 3).

Figure 1. Hatching success (percentage of total hatching in the period of 4 days) of *D. dievengatensis* at SDS and control treatments.

Table 3. Body length (average [μm] $\pm$ SD) of J1 nematodes and at the end of the experiment in SDS treatments and control to *D. dievengatensis* and *L. marina*. Post hoc comparison using *glt* package. a: 0.001% was significantly different of control; b: 0.003% was significantly different of 0.001%; c: 0.006% was significantly different of 0.003%; a', b' and c': 0.001%, 0.003% and 0.006% was significantly different of control, respectively.

Treatment	<i>D. dievengatensis</i>		<i>L. marina</i>	
	J1	End of the experiment	J1	End of the experiment
Control	257.30 $\pm$ 24.14	564.21 $\pm$ 99.35	420.50 $\pm$ 39.58	2151.68 $\pm$ 344.88
0.001%	251.75 $\pm$ 21.11	679.93 $\pm$ 198.90 ^a	457.11 $\pm$ 74.22	1633.91 $\pm$ 347.51 ^{a'}
0.003%	253.68 $\pm$ 21.41	777.64 $\pm$ 222.49 ^b	405.60 $\pm$ 56.51	1610.12 $\pm$ 177.61 ^{b'}
0.006%	237.04 $\pm$ 31.46	591.37 $\pm$ 120.16 ^c	402.80 $\pm$ 52.54	1697.88 $\pm$ 341.08 ^{c'}

a and c = $P < 0.001$; b = $P < 0.05$

a', b' and c' = $P < 0.001$

Figure 2. Growth rate slope of *D. dievengatensis* and *L. marina* . Linear regression lines (solid) plus corresponding 95% confidence intervals (dotted lines) for all data.

Table 4. Pair-wise comparisons of interaction factor (Day*SDS Treatment) of Growth rate and Reproductive rate of *D. dievengatensis* and *L. marina*. *P* values were obtained by *glht* function in R.

	Growth		Rc acumulated	
	<i>D. dievengatensis</i>	<i>L. marina</i>	<i>D. dievengatensis</i>	<i>L. marina</i>
Day:Control x Day:0,001%	0.540	0.002	< 0.001	< 0.001
Day:Control x Day:0,003%	< 0.001	0.052	< 0.001	0.002
Day:Control x Day:0,006%	1.000	0.047	0.193	< 0.001
Day:0,001% x Day:0,003%	0.003	0.531	1.000	0.016
Day:0,001% x Day:0,006%	0.363	0.558	< 0.001	0.010
Day:0,003% x Day:0,006%	< 0.001	1.000	< 0.001	< 0.001

Reproductive rate (Rr), Total fecundity and Survivorship

SDS affected the reproductive rate (*Rr*) of both *D. dievengatensis* ($F= 4.75$ (3), $p= 0.003$) and *L. marina* ($F= 12.04$ (3), $p= < 0.001$). The *Rr* slope of *D. dievengatensis* was significantly higher at SDS 0.001% and 0.003% compared to the control and 0.006% treatment (Fig. 3a, Table 4). The slope of 0.006% SDS did not differ from the control (Fig. 3a, Table 4). Total fecundity for *D. dievengatensis* varied from 16.4 ± 13.8 (average $\pm$ SD) to 40.9 ± 12 and as for the *Rr*, it was significantly higher at SDS 0.001% and 0.003% when compared to the control (Fig. 4a, Table 4). For *L. marina*, *Rr* was significantly lower in all

SDS treatments compared to the control (Fig.3b, Table 4). The highest R_r was observed at the control, followed by 0.003%, 0.001% and 0.006% (Fig. 3b). Total fecundity for *L. marina* was also significantly lower at all SDS treatments compared to the control. However, whereas at SDS 0.001% and 0.003% offspring were produced, at 0.006% total fecundity was practically inhibited (Fig. 4b, Table 4).

The survivorship of adult nematodes from F0 was similar among all treatments for *D. dievengatensis*, independently of the gender (Fig. 5a; Table 6). For *L. marina*, survivorship was affected by exposition to SDS and this effect was dependent on the gender (Table 6; Fig. 5b). Whereas survivorship of females did not differ between treatments, survivorship of males exposed to 0.006% SDS was significantly lower when compared to the control (*post-hoc* $p=0.307$; Fig. 4b).

Figure 3. Reproductive rate slope of *D. dievengatensis* and *L.marina* during 8 day of SDS exposition. Linear regression lines (solid) plus corresponding 95% confidence intervals (dotted lines) for all data.

Table 5. Total fecundity (average [μm] $\pm$ SD) at the end of the experiment in SDS treatments and control to *D. dievengatensis* and *L.marina*. Post hoc comparison using *glht* package. a, b: 0.001% and 0,003% was significantly different of control, respectively; c: 0.006% was significantly different of 0.001% and 0.003%; a' and c': 0.001%, and 0.006% was significantly different of control, respectively; b': 0.003% was significantly different of 0.006%

Treatment	<i>D. dievengatensis</i>	<i>L.marina</i>
Control	18.05 $\pm$ 3.4	73.1 $\pm$ 20.0
0.001%	40.9 $\pm$ 12.4 ^a	21.9 $\pm$ 18.1 ^{a'}
0.003%	37.2 $\pm$ 13.5 ^b	46.5 $\pm$ 31.5 ^{b'}
0.006%	16.4 $\pm$ 13.8 ^c	1.1 $\pm$ 2.3 ^{c'}

a, b and c = $P < 0.001$

a' and b' = $P < 0.05$; c' = $P < 0.001$;

Table 6. Survivorship of parental nematodes (F1) of *D. dievengatensis* and *L. marina* expose to SDS treatment. Generalized linear model (GLM) was used and significance of SDS Treatment, Sex and (Treatment x Sex) effects was tested using likelihood ratio chi-square tests.

	<i>D. dievengatensis</i>			<i>L. marina</i>		
	Df	Deviance Resid	Pr(>Chi)	Df	Deviance Resid	Pr(>Chi)
SDS Treatment	3	2.11078	0.5497	3	2.4892	0.4772
Sex	1	0.21936	0.6395	1	7.0601	0.0078
SDS Treatment x Sex	-	-	-	3	9.4724	0.0236

degrees of freedom [Df]

Figure 4. Survivorship of parental nematodes (F1) exposed to SDS treatment after 8 day. (a) Total survivorship of *D. dievengatensis* and (b) Male and female survivorship of *L.marina*.

DISCUSSION

In this study, the concentrations of SDS tested had negligible effects on the hatching success of the slower species *D. dievengatensis* and no effects on the body size of J1 nematodes immediately after eclosion. In agreement, the post-eclosion body size of the faster species, *L. marina* was also similar between the control and SDS treatments. Total hatching inhibition was only observed for *D. dievengatensis* at SDS concentrations over 0.7%, (Oliveira et al, unpublished data). For both species the integrity of J1 might be a result of the isolation and protection provided by the eggshell, which is composed of an outer vitelline layer, a middle chitin layer, and an inner lipid layer (Foor, 1967; Wharton, 1983; Rappleye et al., 1999). So far, the few data available provide ambiguous evidence that nematode eggshell may both avoid (Lýsek et al., 1985; Mansfield et al., 1992; Bembenek et al., 2007; Benenati et al., 2009) or allow the transport of small molecules inside the eggs (Olson et al., 2012). Given that SDS is a surfactant capable of affecting cell permeability (Laouar et al., 1996), it is suggestive that at high concentrations the eggshell loses its protective property.

Although the sub-lethal concentrations of SDS had no effect on the post-eclosion body size of both species, it significantly affected growth rates and this effect was species dependent. For the ‘slower’ species *D. dievengatensis*, growth rate was highest when exposed the medium concentration of SDS (0.003%) whereas for the ‘faster species’ growth rate was significantly reduced at all SDS concentrations. A possible explanation for such differential response might be related to their differences in energy allocation according to their life-histories. We have estimated energy allocation as the proportion of the female’s body length at maturation, relative to their body length at their full development using data from experimental animals for *L. marina*, and from Table 1 for *D. dievengatensis*. Whereas *D. dievengatensis* allocates about 90% of its energy investment in body size development until reaching maturation, the faster species *L. marina* allocates only 70% to this purpose. Therefore, the decrease in the growth rate of *L. marina* when exposed to SDS may reflect its lower energy allocation to growth and, consequently, lower resistance to stress. This effect could be explained by both a reduction in energy assimilation and/or a reallocation of energy into other physiological processes, such as survivor and reproduction, at the expense of growth (Wicklum & Davies, 1996). Anionic

surfactants are known to interact with various bioactive macromolecules such as starch, proteins, peptides and DNA (Merta & Stenius, 1999; Nielsen et al., 2000) and the exposition to SDS in higher (but still sub-lethal) concentrations has been shown to promote the reduction or inhibition of life-cycle parameters such as growth and reproduction in mussels and terrestrial nematodes (A. Granmo, 1972; Jorge & Moreira, 2005; Harada et al., 2007). Although the direct effects of SDS on organismal growth are not necessarily reflected in altered population dynamics, faster species have been shown to have less competitive advantage at the individual level (Romanovsky, 1984; Aktipis et al., 2013). In addition, *L. marina* still grows after copulation (Tietjen et al., 1970), ensuring a better body condition for the next reproductive events (Aira et al., 2007). Thereby, small changes in growth may have a large effect on future reproduction and hence, on total population fitness.

Increased growth rates, as observed for *D. dievengatensis* at low and moderate concentrations, have already been observed for *C. elegans* juveniles exposed to the surfactant Pluronic (non-ionic polyol block co-polymers) (Mutwakil et al., 1997). This same positive phenomenon was also seen in (i) yeast, due to an increase in cell permeability (Laouar et al., 1996); (ii) plant cells culture of *Solatum dulcamara*, by stimulation of root growth (Kumar et al., 1991; Khatun et al., 1993); and (iii) animal cells cultures of melanoma of hamsters and chick embryonic fibroblasts (Bentley et al., 1989). It has been suggested that the growth-promotion by Pluronic surfactants is a consequence of a mechanism which involves protection from mechanical damage (Handa-Corrigan et al., 1989) and/or an increase in nutrients uptake into cells (Cawrse et al., 1990; Mutwakil et al., 1997). Growth-promotion effects were also described at low SDS concentrations (1-40 ppm) for the macrophyte *Lemna minor* (Dirilgen & Ince, 1995). Such effect was also explained by the use of SDS as a direct source of carbon and/or by an increase in nutrients influx through moderate permeation effects (Lewis, 1991).

Although we were expecting a tradeoff between growth and reproduction as a response to stress (Sibly & Calow, 1989), the trade-off was not evident. SDS contamination affected reproduction and growth on a similar manner. For *D. dievegantensis*, reproductive rate was highest at low and medium concentrations of SDS (0.001% and 0.003%), whereas for *L.marina* it was significantly reduced at all SDS concentrations. For *D. diavengatensis*, in addition to increased growth and reproduction, SDS did not affect survivorship. Such

general increase in organism's performance could be explained as an hormesis effect, which occurs when organisms exposed to lower doses of stress-inducing treatments not merely repair any damage, but also enhance traits associated with fitness (Cypser & Johnson, 2002; Gems & Partridge, 2008; Calabrese, 2015).

For *L. marina*, in addition to decreased growth and reproduction at all SDS levels, survivorship of males was significantly reduced at 'high' levels (0.006%) of SDS contamination. The life history of *L. marina* species is marked by higher energetic investment in reproduction and this strategy could limit the availability of resources for traits like production or maintenance of defense mechanisms against, for example, oxidative stresses (Monaghan et al., 2009; Garratt et al., 2013). The biological effects of SDS have already been related to an increase in surface tension of phosphatidylcholine monolayers, which owing to its tensioactive properties, alter the protection against oxidative stress (Cserhádi et al., 2002). In agreement to our results, susceptibility to oxidative stress may differ between gender, and males with high breeding effort seem to be particularly vulnerable (Alonso-Alvarez et al., 2004).

Despite the lack of a tradeoff between growth and reproduction, the higher survivorship of the 'slower' species *D. dievengatensis* compared to the 'faster' *L. marina* was expected in the light of life history theory. Since these individual level traits are the basis of population dynamics, is important to interpret how individual's response to stress may translate into higher biological organization levels (Jager et al., 2006; Ashauer & Escher, 2010). In an attempt to understand pollution-induced changes in nematode communities, Bongers (1990) developed an index, the Maturity Index (MI), related to nematode's response to stress. This classification uses the characteristics of life-history of nematodes to rank them from 1 (fast species or r strategist) as being most tolerant to disturbances, to 5 (slow species or K strategist) being most sensitive to disturbances (Bongers, 1990; Bongers et al., 1991). *D. dievengatensis* and *L. marina* are both ranked as opportunist nematodes with MI values of 2 and 1, respectively. This classification has been widely used and studies have shown contradictory responses, not necessarily revealing a correlation between this rank and sensibility/tolerance of nematode species to stress (Kammenga et al., 1994; Korthals et al., 1996; Li et al., 2005; Heininger et al., 2007; Brinke et al., 2011; Höss et al., 2011; Ristau et al., 2015). Such contradictory responses may be related to a misinterpretation of how the sensibility/tolerance of nematodes, as

ranked by the MI, corresponds to the different levels of biological organization (i.e. individual vs. population level approach). Nematode species ranked as one (i.e. 'fast' species), as is the case of *L. marina*, are considered resilient, although at the individual level they should be expected to be more sensitive to stress. Such tolerance is a result of the high population growth rate that compensates the injuries or high mortality rate caused by stress. In contrast, nematodes species ranked as 5 ('slow' species) are more resistant to stress at the individual level, but less resilient at the population level, since their life history characteristics, such as low reproductive rates, does not allow a speedy recovery (low resilience). On top of that, the present data shows that species which are relatively close to each other along the fast-slow continuum, responded distinctly to the stress caused by sublethal concentrations of SDS. Therefore caution is needed when scaling up the results from individual level to population and higher organization levels (Fonseca & Gallucci, 2016). Identifying and understanding differential effects of stress in the context of life-history theory is a powerful approach to enhance our understanding and predictive power about the ecological threats posed by anthropogenic activities.

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CONCLUSION

A population of *Diplolaimella dievengatensis* (Nematoda: Monhysteridae) was isolated and cultivated from the Guaratuba river estuary (SP), a relatively pristine environment in the southeast coast of Brazil (SP). This species had never been recorded in this country before. Since free-living marine nematodes have a set of life cycle traits which may suggest low dispersal capabilities, it is necessary to investigate whether species with wide distribution ranges or the cosmopolitans, represent, indeed, complexes of cryptic species. Moreover, considerable cryptic diversity has been observed in the in a wide range of nematode species (DERYCKE et al., 2013; RISTAU; et al., 2015). In chapter one, an integrative taxonomic approach was applied in order to compare individuals of the nematode *Diplolaimella dievengatensis* at distinct life-stages from Brazil with those from the species type-locality, in Belgium coast, described by (JACOBS et al., 1990). The hypothesis that the Brazilian population represented a distinct species within the genus was rejected on the basis of similarities in morphometric and life history traits, as well as 18S gene of the rDNA. Although *D. dievengatensis* from Brazil had 8 of the 32 morphometric parameters isometrically larger than the Belgian population, all diagnostic features were similar. These differences in size were probably related to methodological preparation of measured specimens, or due to inbreeding and demographic stochastic that artificial cultures are subjected.

Considering life history traits, since distinct populations are exposed to different environmental conditions, it could be expected that life cycle parameters here evaluated were affected. In this study, however, both populations exhibited similar performance concerning the parameters measured, for instance in hatching time, final body length and biomass. Slightly differences were observed in the pattern of individual body growth and in the intrinsic rate of natural increase (r_m). The higher r_m in the Brazilian population probably is due to the shorter generation time and higher females' survivorship in this population. Other population parameters were estimated here (e.g. population doubling time, PDT), however comparisons with the local-type population were not possible because of the absence of similar data. Such parameters indicated that populations of *D. dievengatensis* have high productivity since they have potential to double its population in less than two days (PDT=1.48). Other life history traits like high hatching rates, low mortality and ease

maintenance of cultures with minimum laboratorial facilities demonstrated that *D. dievengatensis* is an excellent local model species for further experimental studies. The probable availability of this species in other similar environments (*i.e.* estuaries and coastal lagoons) along the Atlantic Ocean, if confirmed, will make this species an excellent model organism to represent such ecosystems.

Regarding molecular analyses, we found a difference of less than 1% on sequences of 18S rDNA between Brazilian and Belgian animals, which can be attributed to intraspecific variation in marine nematodes (BHADURY et al., 2006). This suggested that the Brazilian species is *Diplolaimella dievengatensis* or belongs to the same species complex. This observation, however, should be further confirmed with the analysis of distinct molecular markers. If conspecific, the ubiquity of this species along the Atlantic Ocean to support the “meiofauna paradox”, in which small benthic organisms with little mobility and without planktonic larval stages can be dispersed over large geographic ranges (Giere, 1993). It was experimentally demonstrated that even weak currents are able to suspend and transport meiofaunal organisms and moreover these are capable of active dispersal into the water column (BOECKNER et al., 2009). In this way, further studies also are necessary in order to investigate possible patterns of population structuration along the species distributional ranges.

A detailed knowledge on life-history traits of *D. dievengatensis* was obtained in chapter one, establishing a baseline which can be useful, in manipulative experiments investigating how biotic and abiotic variables affect such traits. These traits indicate distinct life history strategies among species, which, in turn, demonstrate how these develop and overcome challenges imposed by their environments. The knowledge on the diversity of life-histories can be applied, for instance, in order to identify and understand differential effects of stress posed by anthropogenic activities. In the Chapter two, we investigate how different nematodes species (*Litoditis marina* and *D. dievengatensis*) dealt with stress imposed by sublethal exposition to the sodium dodecyl sulfate (SDS). This pollutant is a residue from innumerable household products, agricultural and industrial activities (SINGH et al., 2007) and it is known to have detrimental effects on the biota (MESSINA et al., 2014). It was hypothesized that the species responses to SDS would be dependent on the particular life history strategies which can be ranked along the “fast-slow continuum” concept (ZERA et al., 2001; RICKLEFS et al., 2002). The *D. dievengatensis* was considered in this study a relatively slower species if compared with *L. marina*, the faster

species. In this way, it was expected that under stress, *D. dievengatensis* prioritize self-maintenance and survival, at the expense of reproduction, therefore individual growth and survivorship rates would be less impacted by SDS exposition. On the other hand, since *L. marina* prioritized reproduction, its growth rates and survivorship would be more affected by the SDS. As expected for the slower species, sublethal exposition did not affected the survivorship rates of *D. dievengatensis*. However, growth and reproduction rates increased significantly at low and intermediate concentrations of SDS (0.001% and 0.003%). This surprising result demonstrated a possible hormesis effect, in which organisms exposed to low levels of toxic substances not only can repair their physiological damages, but also enhance the performance of traits associated with fitness (CYPSEY and JOHNSON, 2002; GEMS and PARTRIDGE, 2008; CALABRESE and BLAIN, 2011). For the faster *L. marina*, survivorship was dependent on adult gender. Survivorship of adult females was not affected by SDS, while for males, the survivorship was significantly reduced only at higher SDS concentrations. We proposed that males might increase their susceptibility to oxidative stress due to the higher breeding effort (ALONSO-ALVAREZ et al., 2004). Also for *L. marina* growth as well as reproduction were decreased in all SDS concentrations. This agrees with the idea that individual from faster species can be more susceptible to physiological injuries, since they allocate fewer resources on defenses and somatic maintenance.

Finally, how different species respond to stress considering the fast low continuum can be interpreted by the “maturity index”, which classify the sensibility/tolerance of nematodes (BONGERS, 1990). However, this classification has a misinterpretation related mainly to level of organization (i.e. individual vs. populational level approach) (FONSECA and GALLUCCI, 2016). The higher tolerance level expected for faster species, as *L.marina*, is a result of their higher population growth, thereby, the resilience of this group is linked to the species response at population and not at individual level. On the other hand, the slower species, as *D. dievengatensis*, can also be considered tolerant to stress, however this resilience occurs at individual level, instead of at population level. This study demonstrated that species which are relatively closely disposed into the fast-slow continuum may have distinct responses to toxic stresses as those caused by the SDS. Moreover, a deeper evaluation of the stress effects considering the life-history theory improves the predictive power about human-induced threats to the environment.

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