



ORIGINAL ARTICLE

The influence of hypo-osmotic stress on the regenerative capacity of the invasive polychaete *Marenzelleria viridis* from its native range

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Abstract

The euryhaline polychaete *Marenzelleria viridis* is a notorious invader of the Baltic region and while many studies have focused on the effects of osmotic stress on larval development of the species, few have assessed its effect on adult specimens. This study investigated the effects of low salinity on the physiology of *M. viridis* by using its regenerative capacity as a proxy for physiologic performance. Specimens were collected in spring 2015 on sandy flats in Hempstead Bay (Long Island, New York), a site that falls within its native range. Worms were ablated between the 14th and 20th chaetigers and cultured at five different salinity treatments (24psu, 15psu, 10psu, 5psu and 0psu) that reflected the broad salinity regimes of its distributional range. Data for anterior regeneration were analysed and presented. Morphogenesis during anterior regeneration was characterized by the formation of a blastema from which the major anatomic structures emerged. All specimens cultured at 5–24psu successfully completed regeneration whereas 75% of specimens cultured at 0psu died and the survivors were unable to proceed beyond the blastema stage. Salinity did not have an effect on regeneration time (14.5–15.1 days) but did have an effect on the percentage of chaetigers regenerated (lowest – 77% at 5psu, highest – 97.5% at 15%). The post-regeneration phase was characterized by variability in pigmentation patterns in the regenerated anterior structures, which appear to be independent of salinity treatments. In conclusion, adult *M. viridis* appears to exhibit high tolerance to low salinity environments, which may have been inherited from its larval stages. In addition, the different pigmentation patterns observed in regenerated structures may allude to the variability of this feature in spionids.

Introduction

Biologic invasions present one of the greatest threats to global marine biodiversity (Pimentel *et al.* 2000; Bax *et al.* 2003; Occhipinti-Ambrogi & Savini 2003; Ricciardi 2007; Molnar *et al.* 2008). In recent years, the issue of understanding the dynamics of marine invasions has received considerable attention as numerous studies have shown that anthropogenic vectors such as ballast water and aquaculture are increasing the rate at which marine

alien species are moved across natural barriers (Ruiz *et al.* 1997; Cohen & Carlton 1998). Most species that are introduced to a new area never actually become invasive, often failing to complete the early stages of the invasion process (survival in the new environment and initial establishment) and some that do become established can be integrated into the community without significantly altering ecosystem functioning (Haydar 2010). However, if a species does become invasive it can have severe ecologic and economic impacts and in some instances, can

negatively affect human health (Bax *et al.* 2003; Pejchar & Mooney 2009).

Constructing the profile of a successful marine invader by identifying key ‘predictor variables’ has been a major goal of bioinvasion studies with the hope that it could help predict future invasion events (Ricciardi & Rasmussen 1998; Kolar & Lodge 2001; Marchetti *et al.* 2004). One particular variable, physiologic tolerance, is believed to be critical when assessing the invasion potential of a species (Berezina & Panov 2004; Marchetti *et al.* 2004; Compton *et al.* 2010; David & Simon 2014). For example, de Rivera *et al.* (2007) used a temperature-dependent approach to successfully predict range expansion of the European shore crab *Carcinus maenas* (Linnaeus, 1758) on the west coast of North America, while Lockwood & Somero (2011) showed that high thermal tolerance of the blue mussel *Mytilus galloprovincialis* Lamarck, 1819, was key to its invasion of the California coast where it has displaced the native *Mytilus trossulus* Gould, 1850.

The polychaete *Marenzelleria viridis* (Verrill, 1873) is one of the best-documented marine invaders, having colonized large areas of Europe (including the Baltic Sea and North Sea; e.g. Atkins *et al.* 1987; Zettler 1997a,b; Bastrop & Blank 2006; Blank & Bastrop 2009; Thomsen *et al.* 2009). The species is native to the east coast of North America, extending as far north as Newfoundland in the north to South Carolina (George 1966; Maciolek 1984) and was most likely introduced to Europe via ballast water (Bastrop & Blank 2006). Since its first discovery in Europe in the Forth estuary, North Sea (Elliot & Kingstone 1987), *M. viridis* has expanded into the Baltic region where it can be found co-occurring with two sibling species, *Marenzelleria arctica* (Chamberlin, 1920) and *Marenzelleria neglecta* (Sikorski & Bick 2004). In these regions, the worms can reach densities of several thousand individuals·m⁻² (Zettler 1996; Zmudzinski *et al.* 1996; Delefosse *et al.* 2012). *Marenzelleria viridis* has significantly impacted its invaded habitat by decreasing the density of some macrobenthic invertebrates and displacing others as a result of inter-specific competition (Kotta *et al.* 2001; Delefosse *et al.* 2012). The success of *M. viridis* in the Baltic Sea is due in large part to its wide salinity tolerance that allows the species to exploit a variety of habitats that would otherwise be uninhabitable for most marine organisms (Stigzelius *et al.* 1997). Field surveys have shown that *M. viridis* is capable of thriving at salinities as low as 5‰ where the species can reach densities of up to 5000 ind.·m⁻² (Zettler *et al.* 1995), while its planktonic larva is able to complete development and metamorphosis at a similar salinity (Bochert *et al.* 1996).

Almost all experimental studies that have investigated the effects of environmental stressors on *Marenzelleria viridis* have focused on larval development (George

1966; Atkins *et al.* 1987; Bochert & Bick 1995; Bochert *et al.* 1996; Bochert 1997) while few have addressed the effects on adults (Fritzsche & Von Oertzen 1995). Although the ability to complete reproduction and larval development is important for survival and initial establishment in an invaded area, long-term establishment and further spread is also dependent on adult performance. Therefore, the purpose of the present study was to investigate the effects of low salinity on adult *M. viridis* by using its regenerative capabilities as a proxy for physiologic performance. Regeneration is a type of post-embryonic development common among annelids, and while it is often associated with asexual reproduction, it also acts as an adaptive strategy to sublethal predation (Bely 2006; Bely & Nyberg 2010; Lindsay 2010). Sublethal predation on *M. viridis* and other spionids is typically due to browsing predators such as fish (Essink & Kleef 1993) and resulting regenerating worms have been known to exhibit abnormalities (e.g. double heads, malformed palps; Stock 1965; Dualan & Williams 2011; David & Williams 2012).

In many polychaete taxa, regeneration involves replacing a specific body part or an entire anatomic region as a result of injury or autotomy (fragmentation; Lindsay *et al.* 2007, 2008; Dualan & Williams 2011). Regeneration is an energetically expensive process that requires significant trade-offs (Lawrence & Vasquez 1996; Pomory & Lawrence 1999; Maginnis 2006; Naya *et al.* 2007; Lawrence 2010) and impacts the feeding biology of spionid worms (Lindsay & Woodin 1992, 1995; Lindsay *et al.* 2008) and as such, it has often been used as a measure of an organism’s physiologic performance (Bely & Nyberg 2010; Freitas *et al.* 2015; Pires *et al.* 2015). *Marenzelleria viridis* is capable of anterior and posterior regeneration (Essink & Kleef 1993; T. Whitford & J. D. Williams, personal observations) as found in a range of other spionids (e.g. Stock 1965; Lindsay *et al.* 2008; Dualan & Williams 2011; David & Williams 2012). In this study, we hypothesized that *M. viridis* exposed to lower salinities would have: (i) higher mortalities compared with the control treatment, (ii) longer regeneration times relative to the control treatment, (iii) fewer regenerated chaetigers at the completion of regeneration compared with the control treatment and (iv) a higher incidence of abnormalities in the regenerated structures compared with the control treatment.

Material and Methods

Marenzelleria viridis was collected from sediment on 29 April 2015 from the northeastern coast of the USA in Hempstead Bay, Long Island, New York (40°50'27.46" N, 73°39'11.70" W). The sampling area was located on

inter-tidal sand flats that are frequently exposed during low tide. Preliminary studies (Williams *et al.* 2016) showed that approximately 7% of worms exhibited regeneration. Sediment was sieved and individual worms along with seawater (24psu; water temperature 15 °C) were sealed in plastic containers and transported to the laboratory at Hofstra University. Worms were examined under an Olympus SZ61 dissecting microscope (NY/NJ Scientific Inc., Middlebush, NJ, USA) for identification. As a consequence of handling, some worms autotomized their posterior ends, and these were isolated into a separate batch and allowed to regenerate.

Artificial seawater solutions were made using Instant Ocean (Cincinnati, OH, USA) diluted with distilled water, including the control treatment (24‰) and four experimental treatments (15psu, 10‰, 5psu and 0psu). The experimental treatment salinity levels were chosen as they encompass the lower range of salinities that characterizes the wide distributional range of *Marenzelleria viridis*. We measured salinity using a refractometer (Milwaukee Instruments Inc., Rocky Mount, NC, USA). Worms (130–180 chaetigers) were placed in Petri dishes at each salinity treatment (12–15 worms-dish⁻¹) and then incubated for 48 h at 15 °C for acclimatization. All worms were cultured without sediment.

After the acclimatization period, worms were anesthetized in 7% MgCl₂ to immobilize the worms for amputations and then ablated between the 14th and 20th chaetigers using a curved scalpel (Table 1). Posterior regions were separated from anterior regions and regeneration of complementary ends was followed at a control temperature of 15 °C. Water changes were carried out twice per week and development was monitored every other day. To investigate specific stages of development, some individuals were arrested by fixation in 3% gluteraldehyde for 2 h at room temperature (21 °C). Specimens were then rinsed in 0.1 M cacodylate buffer (pH = 7.2) three times, each for 30 min. After rinsing, worms were dehydrated in an ascending EtOH series (70%, 75%,

80%, 85%, 90%, 95%) for 10 min for each stage and 100% three times for 15 min each time. Specimens were critical point dried (Samdri-795 Critical Point Dryer), mounted on an aluminum stub with adhesive sticky tape and coated with gold (EMS-550 Sputter Coater), and observed using scanning electron microscopy (FEI Quanta 450 Scanning Electron Microscope). A light microscope (Olympus CX31) with a camera attachment (Olympus DP11) was used to document live specimens. All size measurements were determined using IMAGE J software calibrated by a micrometer slide photographed with the scope (Schneider *et al.* 2012).

In this study, we focused on anterior regeneration because the essential feeding and sensory structures are located on this region of the worm's body. Regeneration was considered 'complete' only after the appearance of the ciliated food grooves on the palps. After regeneration was complete, the post-regeneration period was followed for 7–10 days to detect any variability in morphologic characteristics. A Shapiro–Wilk normality test found that the data did not conform to a normal distribution ($P < 0.05$) and hence a Kruskal–Wallis H test was used to evaluate statistical differences among the different salinity regimes. All statistical analyses were completed using SPSS v. 23 (IBM Corp. Released 2015).

Results

All individuals ($n = 56$) of *Marenzelleria viridis* at 5psu, 10psu, 15psu and 24psu successfully completed anterior regeneration (0% mortality) while high mortality (75%) was observed for worms cultured at 0psu (nine out of 12 worms). In addition, worms that did survive at 0psu failed to progress beyond the blastema stage and hence quantitative data for only four of the five salinity treatments are presented here.

Days 1–5 of anterior morphogenesis were characterized by the formation of a smooth surface at the site of ablation as a result of tissue constriction (Fig. 1A). On day 6, a blastema had formed, replacing the healed tissue (Fig. 1B). Days 7–9 were characterized by extension of the blastema, formation of an early prostomium and the appearance of palp buds. Differentiation of segments, as evidenced by invaginations of the extended tissue on the ventral region (ventral furrows), also occurred during this period (Fig. 1C). The mouth became fully formed on day 10. On days 11–13, palp extension occurred, along with the first appearance of parapodia and inter-segmental septa. Regeneration was completed on days 14–16 and was characterized by fully formed palps with food grooves, nuchal organs and chaetae present on all regenerated segments (Fig. 1D). Under all salinity treatments, regenerated palps were

Table 1. Summary of regeneration experiments on *Marenzelleria viridis* at 15 °C under different salinity treatments.

| salinity (psu) | number of specimens | chaetigers ablated ($m \pm SD$) ^a | chaetigers regenerated ($m \pm SD$) ^b |
|-------------------|------------------------|---|--|
| 5 | 15 | 16.3 ± 1.27 | 12.5 ± 1.91 |
| 10 | 15 | 16.2 ± 1.37 | 13.7 ± 1.76 |
| 15 | 14 | 16 ± 0.39 | 15.6 ± 0.63 |
| 24 | 12 | 15.3 ± 0.99 | 14.7 ± 0.87 |

^aKruskal–Wallis H = 5.09, df = 3, P = 0.165.

^bKruskal–Wallis H = 23.65, df = 3, P < 0.01.

m = mean.

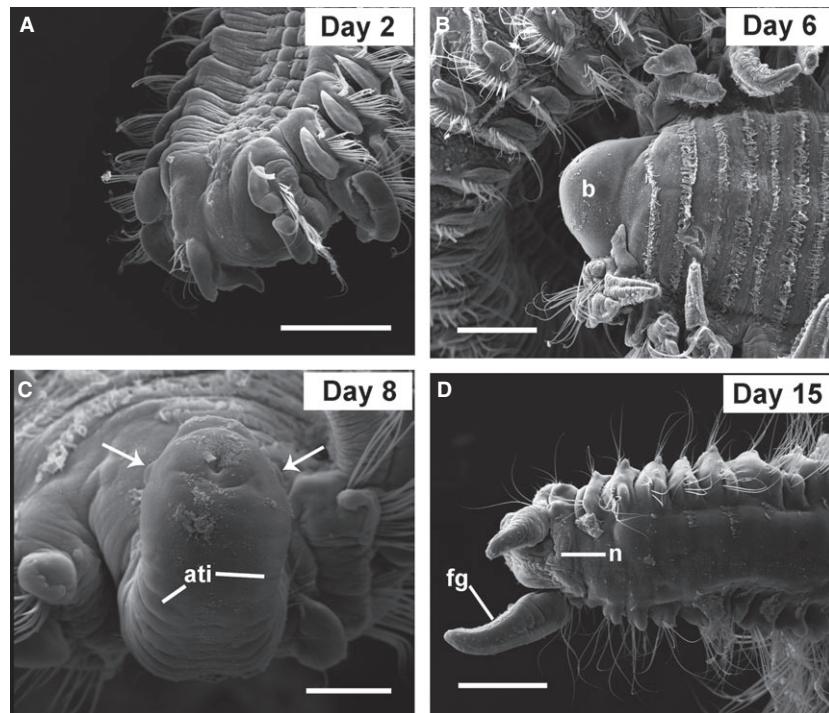


Fig. 1. Scanning electron microscopy images of anterior regeneration in *Marenzelleria viridis* ablated at chaetiger 15 from the control treatment. (A) smooth posterior end 24 h following ablation, (B) anterior blastema (b), (C) extension of blastema with palp buds (arrows) and invagination of the extended tissue and anterior tissue invagination (ati), (D) fully regenerated worm with ciliated food groove (fg) and nuchal organ (n). Scale bars: A = 500 µm, B = 250 µm, C = 150 µm, D = 200 µm.

always shorter than those belonging to non-regenerating adult worms (Fig. 2).

There was no significant difference in the time required for completion of regeneration at 5psu (14.8 ± 0.7 days), 10psu (14.9 ± 0.7 days), 15psu (15.1 ± 0.6 days) and 24‰ (14.5 ± 0.7 days) (Kruskal–Wallis H = 5.28, 3 df, P = 0.15). However, salinity had a significant effect on the percentage of chaetigers regenerated, with worms cultured in the control salinity (24psu) and highest experimental salinity (15psu) regenerating significantly more chaetigers than those at the two lower salinities (5psu and 10psu) (Kruskal–Wallis H = 29.45, 3 df, P < 0.05; Fig. 3). *Marenzelleria viridis* regenerated the highest percentage of chaetigers when cultured at 15psu ($97.5 \pm 3.9\%$) although this was not significantly different from the percentage regenerated at the control treatment of 24psu ($96.2 \pm 4.3\%$). The lowest percentage of chaetigers regenerated occurred at 5psu ($77 \pm 9.8\%$). Worms regenerated a total of 10–16 segments in the 5psu, 10–16 segments in the 10psu, 14–16 segments in the 15psu and 13–15 segments in the 24psu treatment (Table 1).

During the acclimatization period prior to ablation, the feeding palps of all specimens cultured at the lower salinities (5psu and 10psu) became turgid (Fig. 4A). However, the palps developed ‘normally’ after regeneration was completed (based on formation of ciliated food grooves as examined with scanning electron microscopy) and no abnormalities were found during the post-regen-

eration period (Fig. 4B). During the post-regeneration period (days 16–21), the branchiae formed and approximately 42% of animals developed variable epidermal pigmentation patterns (Fig. 5). Despite the variability, the pigmentation patterns could be grouped into three types; type 1 possessed pigmentation concentrated on the prostomium and peristomium, type 2 had pigmentation extending along the dorsal midline of the anterior chaetigers while type 3 had very faint pigmentation with no discernable pattern. Salinity did not appear to influence pigmentation because all three patterns could be found in a single salinity treatment.

Discussion

In this study, we investigated the effects of salinity levels on the regenerative capacity of *Marenzelleria viridis*. The results showed a broad salinity tolerance, with all worms surviving at salinities of 5–24psu. However, a salinity of 0psu resulted in a very high mortality rate (>50%) and worms that did survive were unable to regenerate any tissue beyond blastema formation. Interestingly, salinity did not affect the timing of regeneration but lower salinities did result in a lower percentage of chaetigers regenerated. In addition, regenerated anterior ends (prostomium and peristomium) exhibited variation in pigmentation patterns during the post-regeneration phase, which appeared to be independent of the salinity treatments.

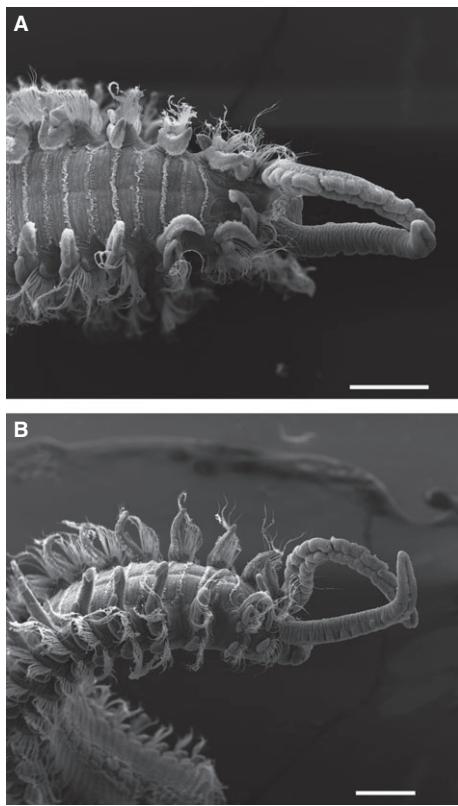


Fig. 2. Scanning electron microscopy images of *Marenzelleria viridis* showing (A) anterior dorsal and (B) anterior lateral views of an intact non-regenerating adult. Scale bars: A = 500 µm, B = 500 µm.

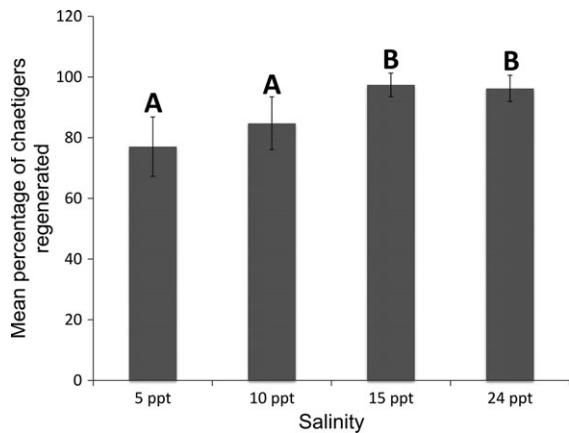


Fig. 3. Effect of salinity on the percentage of chaetigers regenerated in *Marenzelleria viridis* at five different salinity treatments. Whiskers represent standard deviation (SD); letters denote significant differences between treatments using an independent-samples Kruskal–Wallis test (significance, P < 0.05).

In marine invertebrates, salinity tolerance varies considerably among species (Topping & Fuller 1942; Lyster 1965) and is believed to be one of the key physiologic

attributes that determine invasion success in the marine realm (Ricciardi & Rasmussen 1998; Mann & Harding 2003; Minchin *et al.* 2009; Capps *et al.* 2011). Based on the results from this study, we accept hypothesis i (higher mortalities of worms in low salinities compared with the control treatment) for the 0psu salinity treatment. The high mortalities and arrested regeneration of *Marenzelleria viridis* at 0psu are congruent with larval developmental studies on the species. Larvae cultured at salinities below 5psu showed high mortalities and those that did survive were unable to complete development beyond the three-chaetiger stage (George 1966; Bochert 1997). One important implication of these findings is that the broad salinity tolerance of *M. viridis* could be inherited across different ontogenetic stages of the species' life cycle. In fact, Bochert (1997) found that salinity tolerance of *M. viridis* larvae was only 'slightly' lower than the juveniles and adults. This stands in contrast to other studies on decapods and gastropods, which exhibit distinct differences in salinity tolerance between larvae and juveniles, and juveniles and adults (Mann & Harding 2003; Bravo *et al.* 2007). A high salinity tolerance in *M. viridis* inherited across life-history stages could therefore be one explanation for the pre- and post-settlement success of the species in meso- and oligohaline habitats. Future garden experiments coupled with follow-up regeneration studies on *M. viridis* should provide evidence for such a hypothesis.

Morphogenesis during anterior regeneration in *Ma. viridis* followed a similar trend to other spionids, with formation and extension of a blastema from which the major anatomic features arose (Gibson & Harvey 2000; Gibson & Paterson 2003; Lindsay *et al.* 2007; David & Williams 2012). However, regeneration time did not differ across salinities and hence we reject hypothesis ii (longer regeneration times for lower salinities relative to the control treatment). We expected regeneration times to be longer at lower salinities due to the added time required for osmotic regulation. Furthermore, these experiments were not carried out using a gradual acclimatization protocol and hence the large dilution intervals (*i.e.* 24–5psu) should have added further delays. Our findings are in contrast to studies on other invertebrate taxa such as decapod crustaceans and onuphid polychaetes that have shown delayed developmental times as a result of hypo-osmotic stress (Anger *et al.* 1998; Freitas *et al.* 2015). One explanation for our findings is that the rate of osmotic regulation may have remained constant at the different salinity treatments, which would be possible if the population sampled was locally adapted to wide fluctuations in salinity (*e.g.* periodical freshwater input). Freshwater inputs are found at the sampled site in Hempstead Bay site, through run-off from nearby

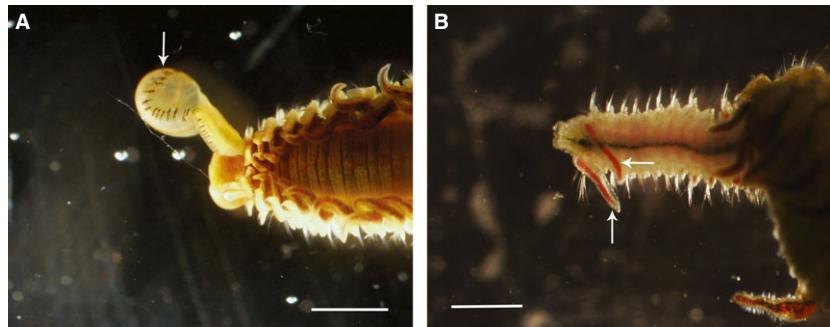


Fig. 4. (A) Turgid palps (arrow) in *Marenzelleria viridis* cultured at 5psu prior to ablation, (B) 'normal' palps at 5psu (arrows) regenerated 15 days post-ablation. Scale bars = 0.5 mm.

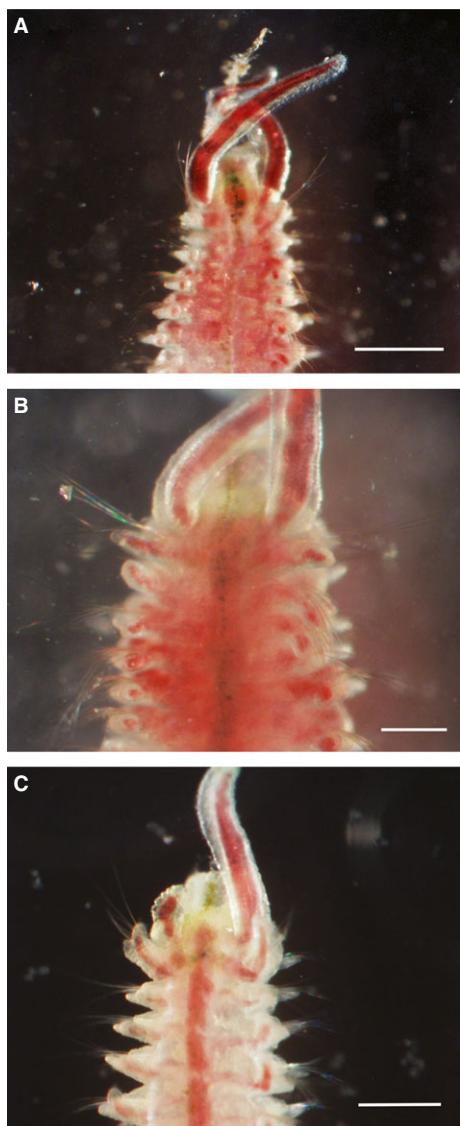


Fig. 5. Variable pigmentation patterns in *Marenzelleria viridis* among regenerated anterior tissue during the fifth day of the post-regeneration period (20 days post-ablation). (A) type 1, (B) type 2, (C) type 3. Scale bars: A, B = 0.5 mm, C = 0.25 mm.

roadways and a brackish pond (authors 'personal observations).

There was a decrease in the mean percentage of chaetigers regenerated at 5psu and 10psu compared with at 15psu and 24% and hence we failed to reject hypothesis iii (lower number of regenerated chaetigers in low salinities compared with the control). In this case, the worms cultured under low salinities may have reduced the number of chaetigers regenerated by adjusting their osmolarity. This trade-off of reduced growth rate in response to hypo-osmotic stress has been well documented in other marine invertebrates ([Anger et al. 1998, 2000](#); [Jahnke & White 2003](#); [Riisgard et al. 2012](#)) but this is the first time that it has been demonstrated for tissue regeneration specifically for *Marenzelleria viridis*. It appears that some spionids regenerate a fixed number of segments following ablation (seven to 13 segments in polydorids and *Pygospio*; [Stock 1965](#); [Lindsay et al. 2007](#); [Dualan & Williams 2011](#)). In this study, *M. viridis* was capable of regenerating up to 10–16 segments following ablation.

There were no observed abnormalities in regenerated structures and hence we reject hypothesis iv (higher incidence of abnormalities in low salinity treatments compared with the control). The large dilution gap used in this study was most likely responsible for the turgid palps in specimens at the lower salinities. Turgidity in anatomic structures as a result of hypo-osmotic stress has been found in marine invertebrates ([Topping & Fuller 1942](#); [Held & Harley 2009](#)). However, regeneration of 'normal' palps only 7 days after ablations is strong evidence supporting rapid adaptation of *Marenzelleria viridis* to the large shifts in salinity regimes. Alternatively, the palps of spionids may be deciduous and can be easily regenerated regardless of environmental conditions.

The differences in pigmentation patterns in *Marenzelleria viridis* during the post-regeneration phase were unexpected. Because pigmentation varied considerably across salinity treatments and even within specific treatments, it is unlikely that salinity was a causative factor. Instead, our results indicate that pigmentation patterns in *M. viridis* could be a variable trait, although we did not

follow long-term growth and thus do not know exactly how pigmentation patterns will ultimately be exhibited. This has implications for taxonomic verification of the species due to the fact that previous diagnoses have included pigmentation ‘spots’ and patterns as part of the general description of *M. viridis* (Atkins *et al.* 1987; Sikorski & Bick 2004). In fact, a comprehensive morphologic study by Sikorski & Bick (2004) asserted that pigmentation was a characteristic trait of *M. viridis*, although the authors admitted that ‘nothing is known about variation in this character’. Here we show that pigmentation pattern is highly variable in regenerated anterior structures and because regeneration may occur frequently in benthic habitats due to sublethal predation, this trait could lead to unnecessary taxonomic confusion. A recent genetic study on *Polydora hoplura* also demonstrated the highly variable nature of pigmentation in spionid worms (van Niekerk 2014). Future experiments should focus on culturing the worms over a longer time scale to determine if pigmentation patterns change long after anterior regeneration is completed.

Finally, climate change is expected to cause alterations in rainfall patterns that would induce shifts in the salinity regimes of the world’s oceans (Reid *et al.* 2003; Cardoso *et al.* 2008). More specifically, future scenarios predict heavy rainfall events that would lead to hypo-osmotic stress for many estuarine animals (Bussell *et al.* 2008). This study supports previous work that shows that *Marenzelleria viridis* possesses an impressive ability to rapidly acclimatize, adapt and thrive under these conditions. As such, predicted decreases in ocean salinity levels may in fact be advantageous to *M. viridis* in the northern seas due to reduced competition by stenohaline benthic invertebrates like amphipods and a wide variety of polychaetous annelids (Kotta *et al.* 2001; Kotta & Olafsson 2003; Neideman *et al.* 2003).

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