

LRH: Licciano, Watson, Murray, & Giangrande
RRH: Regenerative ability of *Myxicola infundibulum*

**Evidence of regenerative ability in *Myxicola infundibulum*
(Annelida, Sabellida): evolutionary and systematic implications**

Margherita Licciano,^{1,3} Gordon James Watson,²
Joanna Michelle Murray,^{2,3} and Adriana Giangrande¹

¹Department of Biological and Environmental Sciences and Technologies,
University of Salento, 73100 Lecce, Italy

²Institute of Marine Sciences, School of Biological Sciences,
Eastney, Portsmouth PO4 9LY, UK

³Centre for Environment, Fisheries and Aquaculture Science,
Lowestoft, Suffolk NR33 0HT, UK

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^aAuthor for correspondence. E-mail: margherita.licciano@unisalento.it

Abstract. Members of only a few species of annelids are reported as being incapable of regeneration; of these, *Myxicola infundibulum* is the only example in the family Sabellidae. Interestingly, its congener *M. aesthetica* exhibits noteworthy regenerative ability. Unambiguously identifying non-regenerating species is critical to reconstructing how regenerative abilities evolved within the phylum. However, studies designed specifically to assess the regenerative potential of *M. infundibulum* have never been performed. The current study aimed to confirm the lack of regeneration ability of *M. infundibulum*, previously reported for Atlantic specimens, or to determine the extent to which regeneration occurs. Our results showed that individuals from the Mediterranean Sea (Adriatic Sea) do undergo regeneration of lost body parts, although to a lesser extent than found among other sabellids. Therefore *M. infundibulum* should no longer be considered a non-regenerating species. At present, uncertainties regarding phylogenetic relationships of Sabellidae prevent inferences about the polarity of change in *M. infundibulum*. Since our findings are counter to those of previous studies which describe Atlantic specimens as non-regenerating, more extensive analysis is required to ascertain if they could actually belong to a different species than Mediterranean *M. infundibulum*, accounting for these differences in reported regenerative capacity.

Additional key words: evolution, regeneration, Sabellidae, systematics

Annelida are well known for their impressive regenerative abilities. Depending on the frequency of injury and capacity for regeneration, these abilities not only provide an immediate survival advantage to the individual, but can also have impacts on populations, communities, and ecosystems (Zajac 1985, 1995; Lindsay & Woodin 1995; Lindsay et al. 2007, 2008; Lindsay 2010). Evidence for interspecific variation in annelid regenerative abilities has been extensively documented, with some species able to reconstitute an entire individual from a single body segment, and others completely incapable of regeneration (Morgulis 1907; Okada 1929; Hyman 1940; Berrill 1952; Herlant-Meewis 1964; Bely 2006). Posterior segment regeneration appears to be nearly universal within the phylum, while the ability to regenerate anteriorly is common but less widespread (Bely 2006, 2010; Brookes & Kumar 2008; Zoran 2010). By mapping this information onto a molecular phylogeny for the

group, Bely (2010) found that annelid regeneration is an evolutionary labile feature which has been gained or lost multiple times.

A number of studies provide clear evidence on the presence of regenerative ability in many species of annelids, but studies that report the absence of regenerative ability typically relies on anecdotal observations or unpublished data (Bely 2006). Observations of the lack of regeneration have often been interpreted as inconclusive, uninteresting, or simply not worth publishing (Bely 2006, 2010). However, identifying non-regenerating species and examining data on the presence/absence of regeneration in a phylogenetic context represents a powerful method by which to elucidate the pattern of evolution of regeneration (Bely 2010). Providing strong evidence for the absence of regenerative ability, however, is more difficult than demonstrating the presence of this ability. While the presence of regeneration is proved unambiguously if successful regeneration ensues, failure to regenerate does not necessarily indicate “absence” of this ability, and further studies must then be performed under a broad range of experimental conditions (Bely, 2006, 2010; Bely & Sikes 2010). This approach, however, has rarely been followed, and rigorous studies are needed not only to identify new non-regenerating species but also confirm absence of regenerative ability in some previously identified non-regenerating species.

In a recent review on the distribution of segment regeneration ability in annelids, only a few taxa were been reported as being incapable of any regeneration, either anteriorly or posteriorly: Hirudinida, Branchiobdellida, and the polychaetes *Arenicola marina* (LINNAEUS 1758) (Arenicolidae), *Polyophthalmus pictus* (DUJARDIN 1839) (Opheliidae), and *Myxicola infundibulum* (RENIER 1804) (Sabellidae) (Bely 2006). Among them, *A. marina* only regrows its tail by lengthening remaining tail segments, not by adding new segments (De Vlas 1979), while *P. pictus* is only capable of posterior wound healing (Hyman 1940). The absence of any regeneration in the sabellid *M. infundibulum* deserves particular attention, as this species is the only member of the Sabellidae alleged to lack regenerative ability. Anterior and posterior regeneration has been documented for the other seven species of sabellids thus far examined (Berrill 1931; Gross & Huxley 1935; Berrill & Mees 1936; Berrill 1952; Wells 1952; Bely 2006; Murray 2010; Licciano et al 2012; Murray et al 2013), including *M. infundibulum*'s congener *Myxicola aesthetica* (CLAPARÈDE 1870), which showed powers of regeneration so remarkable that any segment of the body could give rise to a new “head” anteriorly and a new “tail” posteriorly (Okada 1932, 1934). In contrast to its congener, however, no investigations of the regenerative potential of *M. infundibulum* have been performed. The only information on regeneration in this species is available from a paper dealing with the respiratory significance of the crown in sabellids (Wells 1952). In this paper, a footnote briefly reports unpublished data on the failure of any regeneration process in specimens of *M. infundibulum* from Plymouth during some experiments performed by Nicol (in Wells 1952).

In order to either unequivocally confirm the lack of regeneration in *M. infundibulum* or determine the extent to which individuals can restore lost body parts, we investigated the regenerative potential of specimens collected from the Mediterranean Sea (Southern Adriatic Sea, Italy) after different cutting treatments performed along the body axis. The morphology of the regeneration process of anterior and posterior ends, regeneration timing, and mortality were evaluated in different types of body fragments (including mid-body fragments) resulting from specific artificial amputations.

Methods

Study species and specimen collection

Members of *Myxicola infundibulum* are sedentary, living in thick, gelatinous tubes almost completely buried in soft sediments. The cylindrical bodies of the individuals of this

infaunal species are comprised of serially repeated segments (chaetigers), each separated by septa. The body has distinct thoracic and abdominal regions. The thorax typically consists of eight chaetigers, whilst the abdomen comprises a variable number of chaetigers (up to 100). Anteriorly, the prostomium is modified into a purple or brown branchial crown used in feeding and respiration; it forms a characteristic funnel, with tentacles joined for most of their length by a palmate membrane, and with just the tips free. The mouth is terminal, on the peristomium. At the posterior end, the body terminates with the pygidium, which bears the anus. As sabellids grow by continuous addition of new chaetigers derived from the prepygidial growth zone, the youngest body segment is always the most posterior (Schroeder & Hermans 1975; Rouse & Pleijel 2001).

For the present work, specimens of *M. infundibulum* were collected during November 2013 at 5 m depth in a single collection by SCUBA divers from the Southern Adriatic Sea (Brindisi, Italy) (Fig. 1A,B). Within 1 h after collection, worms were transferred to the laboratory and acclimatised in an aquarium held in a room set at 20°C for at least 3 d prior to regeneration experiments to allow recovery from collection and to assess health. It should be noted that within the acclimatization period in aquaria, some worms spontaneously spawned. Further, during amputation experiments, the coelomic cavity was found full of mature gametes in some individuals but devoid of germinal products in others. Analysis of coelomic contents of all the worms enabled these two groups to be separated for regeneration experiments, each performed according to the same experimental procedures described below. Regeneration experiments were run in parallel for all the individuals (with and without gametes) and treatments (1-cut treatment and 2-cut treatment).

Regeneration experiments

Prior to cutting treatments, 90 worms of similar size (90-100 chaetigers), 45 containing gametes and 45 devoid of gametes, were removed from their tubes. For each experiment (i.e. within each set of 45 worms), a first group of 15 worms were cut transversely once within the abdominal region using a razor blade (1-cut treatment) in order to obtain body fragments of the following categories (Fig. 2A):

A1 – an anterior fragment including the branchial crown, all thoracic, and some abdominal chaetigers (total of about 45 chaetigers). Successful regeneration in this fragment would require healing and regeneration of the pygidium and new abdominal chaetigers from a single cut surface.

P1 – a posterior fragment including the remaining abdominal chaetigers (about 45 chaetigers) and pygidium. Successful regeneration in this fragment would require healing and regeneration of the branchial crown and thoracic chaetigers from a single cut surface.

A second group of 15 worms were cut twice transversely within the abdominal region (2-cut treatment) so that body fragments of the following categories were obtained for each worm (Fig. 2B):

A2 – an anterior fragment which included the branchial crown, all thoracic, and some of the abdominal chaetigers (total of about 30 chaetigers). Successful regeneration in this fragment would require healing and regeneration of the pygidium and new abdominal chaetigers from a single cut surface.

M – a mid-body fragment including only abdominal chaetigers (about 30 chaetigers) and with wounds at anterior and posterior ends. Successful regeneration in this fragment would require healing and regeneration from two cut surfaces: anteriorly, the branchial crown and thoracic chaetigers, and posteriorly, the pygidium and new abdominal chaetigers.

P2 – a posterior fragment which included remaining abdominal chaetigers (about 30 chaetigers) and pygidium. Successful regeneration in this fragment would require healing and regeneration of the branchial crown and thoracic chaetigers from a single cut surface.

Finally, a third group of 15 uncut worms were used as controls for mortality. In order to determine the presence/absence of gametes in these uncut worms, a small quantity of coelomic fluid was withdrawn from the body wall using a microsyringe and examined with a compound microscope for the presence of gametes.

After cutting treatments, all body fragments (15 A1, 15 P1, 15A2, 15 M, and 15 P2, for a total of 75 regenerating body fragments for each experiment) and uncut control worms (15 for each experiment) were housed individually in 1 L beakers (total of 90 beakers for each experiment) held in a temperature controlled room at 20°C. Each beaker contained continuously aerated filtered seawater (0.22 µm pore size) which was changed daily. At each water change, liquid food for filter feeding marine invertebrates (SERA Coraliquid) was added at the concentration recommended by the manufacturer (25µL L⁻¹).

In order to assess survivorship and regeneration of missing body parts, all the body fragments were monitored daily using a stereomicroscope equipped with a digital camera (Nikon Coolpix 990, Nikon Corporation, Tokyo, Japan). Following Murray et al. (2013), live fragments were identified as those contracting and relaxing when stimulated, having structurally intact chaetae and, for anterior fragments, the ability to open the branchial crown.

For each fragment type, experimental observations lasted until 100% mortality was recorded. Uncut control worms were maintained in the above described laboratory conditions until 100% mortality was recorded for all body fragment categories (about 7 months)

Results

Body fragment survivorship

Survivorship of each body fragment type over time is shown in Fig. 3 (A-F). Survival of M fragments was the lowest among the examined body piece categories. A1 fragments exhibited the highest survivorship, regardless of the presence/absence of gametes, while P1 fragments showed higher survival than P2 fragments.

1-cut treatment. Survivorship of A1 fragments of individuals without gametes decreased to 67% about 50 days after cutting, a value which held steady for at least another five months (Fig. 3A). When 1-cut treatments were performed on individuals with gametes, survival of A1 fragments decreased to 33% on day 8. This value remained steady for the ensuing five months. Low survivorship was observed for P1 body fragments with gametes, which experienced 100% mortality by day 8 (Fig. 3B). At the same time, P1 fragments of individuals without gametes showed survival of 33%, a value recorded until about two months later, when they experienced 100% mortality.

2-cut treatment. When 2-cut treatments were performed, all body fragments showed low levels of survival. Independent of the presence/absence of gametes, 100% mortality was recorded for all the body piece categories by day 8 (Fig. 3C-E). However, fragments of worms with gametes showed rapid decline in survivorship in comparison with fragments from worms without gametes. In particular, M fragments, both with and without gametes, did not survive beyond 4 and 6 d respectively (Fig. 3E), and were not able to start any regenerative event, including wound healing.

Uncut control worms. In both the experiments, uncut control worms were maintained under the same experimental conditions above described for about 7 months, with less than 10% mortality, regardless of the presence/absence of gametes (Fig. 3F).

Wound healing

The initial repair phase of body fragments occurred during the first 24-96 h after cutting. In all body fragments, circular muscle fibers near the amputation site at anterior and posterior ends contracted, constricting tissue around the cut surface and allowing the wound to close (Fig. 4A). Later, a thin layer of epithelium covered the wound and the healing process was complete. In unhealed fragments, the initial tissue constriction near the cut surface did not occur, leading to the failure of the wound to close.

1-cut treatment. All A1 fragments achieved wound healing. However, anterior fragments of individuals without gametes healed more quickly than fragments with gametes (24 vs 48 h). By contrast, only 33% of P1 fragments with gametes and 75% of P1 fragments without gametes healed. P1 fragments of individuals without gametes healed more rapidly than P1 fragments with gametes (24 vs 48 h).

2-cut treatment. Only anterior and posterior body fragments obtained from 2-cut treatment individuals, regardless of absence or presence of gametes, achieved wound healing 4 d after cutting. Successful wound healing was more common in A2 and P2 fragments without gametes compared to those with gametes (60 vs 20% for A2 and 60 vs 40% for P2). All M fragments died before the process of wound healing began.

Regenerative development

Wound healing was followed by the development of a regenerative bud (blastema) at the posterior and anterior wound surfaces of anterior and posterior body fragments, respectively.

In posterior body fragments (P1 or P2), the anterior blastema appeared within the invagination caused by tissue constriction during anterior wound healing. No difference in timing was recorded for this body piece to develop depending on presence/absence of gametes. However, onset of the anterior regenerative bud was observed in all P1 fragments 4-18 d post-treatment, whilst only 2% of P2 fragments formed a blastema before 100% mortality by day 6. As a consequence, further development of the anterior blastema was observed only in P1 fragments.

In 60% of P1 fragments, the anterior regenerative bud further developed into a rudimentary branchial crown emerging from the invagination of the cut surface, initially consisting of a structure with crenulated outer margins (Fig. 4B,C). At day 15, at least three rounded lobes could be distinguished on the developing crown (Fig. 4D); these could be recognized as radioles by day 35 (Fig. 5A). Subsequently, the crown increased in length and number of radioles, and gained pigmentation (Fig. 5B). Finally, at day 60, a ventral extension of the anterior peristomial ring was also detectable (Fig. 5C). Although observations lasted over five months, no further morphological changes occurred. The regeneration of the anterior end in posterior body fragments of *Myxicola infundibulum* thus stopped about two months after the initial amputation, and never proceeded to a complete, fully functioning thoracic region and branchial crown. Posterior body fragments were able to make new tubes when they were removed from their previous tubes for observations (Fig. 5D).

The onset of regenerative bud development began 4-8 d post-amputation among anterior body fragments, regardless of the presence/absence of gametes. However, because of 100% mortality by day 8 for A2 fragments, further development of the posterior blastema was observed only in A1 fragments. The posterior blastema in these fragments gave rise to a rudimentary anal opening (Fig. 6A), then gained dark pigmentation (Fig. 6B), and finally developed into a pygidium after 15 and 10 d in fragments with and without gametes, respectively (Fig. 6C,D). Abdominal chaetigers were added sequentially at the posterior end and by day 44, 4-6 unpigmented, newly formed chaetigers could be detected in the prepygidial growth zone of fragments without gametes (Fig. 6D). Among individuals with gametes this process was slower, and new abdominal chaetigers appeared at 50 d after

amputations. Anterior body fragments were able to make new tubes when they were removed from their previous tubes for observations (Fig. 6B).

Discussion

Myxicola infundibulum has been described as the sole member of Sabellidae that is incapable of regeneration of segments (Wells 1952; Bely 2006). Wells (1952) stated that if the crown was partially removed, leaving the bases of the radioles, the radiolar bases formed new tips as well as palmate membrane so that worms rapidly (within two weeks) regenerated small but fully formed crowns. However, if the crown was removed along with one or two anteriormost segments, worms rapidly died. Nicol (unpublished data in Wells 1952) also reported that if worms were bisected at the junction of thorax and abdomen, the anterior body fragments lived for weeks without any sign of posterior end regeneration, while posterior body fragments died in a few days. In contrast, our findings confirm that *M. infundibulum* is able not only to regenerate new body segments posteriorly, but also to regenerate the branchial crown even when the branchial crown, the eight thoracic chaetigers, and a variable number of abdominal segments were removed. The regenerative capacity of *M. infundibulum* does, however, seem to be lesser than that of other previously investigated sabellids. Posterior body fragments only regenerated a branchial crown and a small peristomial ring, not thoracic or abdominal segments. Mid-body fragments were not able to heal wounds or undertake any regeneration posteriorly and anteriorly, and indeed suffered 100% mortality. Anterior body fragments were the only body fragment type able to complete regeneration and restore the morphology of individuals. The failure of posterior fragments to regenerate thoracic chaetigers has not previously been reported before within Sabellidae, although posterior body fragments of several species of *Sabellastarte* and *Branchiomma nigromaculatum* BAIRD 1865 cannot regenerate a full complement of thoracic chaetigers (Berrill 1978; Murray et al 2013). Unlike these exceptions, other examined species within Sabellidae have demonstrated substantial regeneration ability and high survivorship of body fragments after amputations. Anterior and posterior body fragments from *Bispira melanostigma* (SCHMARDA 1861) (Berrill 1978), *Branchiomma luctuosum* GRUBE 1869 (Licciano et al. 2012), *M. aesthetica* (Okada 1932, 1934; Caullery & Mesnil 1920), *Pseudopotamilla reniformis* (BRUGUIÈRE 1789) (Kolbasova et al. 2013), *Sabella pavonina* SAVIGNY 1820 (Murray et al. 2013), and *Sabella spallanzanii* (GMELIN 1791) (Licciano et al. 2012) are able to complete regeneration and regain original morphology. Similar findings have been reported for mid-body fragments of *S. spallanzanii*, *S. pavonina* and *M. aesthetica* (Okada 1932, 1934; Licciano et al. 2012; Murray et al. 2013). The finding of differential results with the regenerative abilities of different body fragments of *M. infundibulum* is particularly interesting and is worthy of further research. According to Fitzharris (1973, 1976) there is evidence to support the hypothesis that sabellid regeneration and the expression of polarity during morphogenetic events are under neural control. However, while it is well known that synthesis, packaging, and transport of neurosecretory material produced by the supraoesophageal ganglion play a role in regulating posterior regeneration of some species of *Nereis* and *Nephtys* (Clark & Clark 1959; Clark & Scully 1964; Golding 1967), very few data on the physiological control of sabellid regeneration are available. Kiortsis & Moraitou (1965) proposed a model involving interaction of gut and nerve cord in posterior regeneration in *S. spallanzanii*, similarly to what suggested by Fitzharris & Lesh (1969) for anterior regeneration in *B. melanostigma* and *B. nigromaculatum*. In this last species, however, the ventral ganglia rather than the brain are important in posterior regeneration (Hill 1972). On these grounds and taking into account that simultaneous anterior and posterior regeneration occurs in many of the investigated sabellids, this topic merits further investigation.

Following removal of the anterior end, posterior body fragments were able to regenerate the branchial crown and a small anterior peristomial ring by ~60 d after amputation. The original shape and size of the crown was, however, never attained through regeneration despite individuals remaining in good health for about 5 months. This interruption of regeneration in an advanced phase has rarely been reported. In non-regenerating annelids, regeneration generally appears to be blocked about the time of blastema formation (Bely 2010). Energetic tradeoffs between regeneration and other processes (e.g. growth, reproduction) are common (Maginnis 2006; Bely 2010; Lawrence 2010) and have already been suggested within sabellids for *B. luctuosum* (Licciano et al. 2012) and *P. reniformis* (Kolbasova et al. 2013). It could be hypothesized that following an initial energetic investment in regeneration, the energetic reserves in *M. infundibulum* amputees could have been insufficient to offset the energy required to complete regeneration of the crown. We suggest that for this species, energetic investment in respiration at the expense of regeneration could account for incomplete crown reconstitution. In all previously investigated sabellids, the branchial crown is the first structure to form in anteriorly regenerating fragments, which may be related to its essential role in feeding and respiration (Berrill 1931; Okada 1934; Giangrande 1991). It has been shown, however, that some species drive water through their tubes by waves of muscular contraction running along their bodies to meet respiratory needs, therefore the effect of crown removal in sabellids triggers differences in the respiratory response of different species (Berrill 1931; Zood 1931; Okada 1934; Wells 1952; Giangrande 1991). Wells (1952) showed that individuals of *M. infundibulum* depend largely on the crown for their oxygen supply, as the body is stout, with a thick and glandular skin; the body is in a tightly fitting gelatinous tube; and there is no irrigation current through the tube. This could explain the low survivorship and slow speed of regeneration recorded for all the body fragment types, and the relatively higher survival exhibited by the anterior body fragments, which retained the original crown. Interestingly, our study also found that fragments taken from individuals of *M. infundibulum* with gametes showed higher mortality values and a lower rate of regeneration when compared to fragments obtained from individuals without gametes. Although identifying the underlying drivers for a relationship between reproduction and regeneration in *M. infundibulum* falls outside the aim of the present research, it could be hypothesized that the higher metabolic needs of ripe individuals compared to unripe ones may account for the regenerative patterns observed in this species. Similar findings have been reported for *B. luctuosum* and *P. reniformis* (Licciano et al. 2012; Kolbasova et al. 2013), whose survivorship during regeneration is likely to have been affected by the reproductive status of the worms which devote most of their energy budget to reproduction.

Regeneration provides obvious benefits to an injured individual, yet regenerative abilities appear to have been greatly restricted or completely absent in many animal lineages (Bely & Nyberg 2010; Giangrande & Licciano 2013). The phylogenetic distribution of anterior and posterior regenerative abilities across annelid groups suggests that regeneration may be an ancient trait and that multiple losses and/or gains have likely occurred during annelid evolution (Bely 2010). The ability to regenerate posteriorly is very common, and is possibly related to the mechanism of continual adult growth by posterior segment addition (Herlant-Meewis 1946; Bely & Wray 2001; Bely 2006). All taxa incapable of posterior regeneration are incapable of anterior segment regeneration, while taxa with posterior regeneration may not do so anteriorly. Several non-regenerating species are closely related to regenerating species, suggesting evolutionary losses or gains (Bely 2006; Brookes & Kumar 2008). The present study showed that individuals of *M. indundibulum* can regenerate body segments posteriorly but not anteriorly, in contrast to the closely related *M. aesthetica*, where both these abilities are present. By mapping available data onto a molecular phylogeny for

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the group from Struck et al. (2007), Bely (2010) found that absences of regeneration are not phylogenetically restricted but rather are scattered across the phylum, with seven annelid families including both regenerating and non-regenerating species. This group included Sabellidae, owing to the reported absence of regeneration in *M. infundibulum*, as opposed to the presence of regeneration in *M. aesthetica*. The absence of regeneration in *M. infundibulum* was hypothesized to represent loss of this ability by Bely (2006), who however acknowledged that uncertainties about the deep-level phylogeny of annelids made strong inferences on ancestral character states difficult. Brockes et al. (2001) noted that the absence of regeneration in closely related species is commonly regarded as loss, assuming that regeneration is a primordial attribute of metazoans, and that losing this ability is more likely than gaining this feature. According to these authors, these inferences are based on little direct evidence, and the limited knowledge about the relative probability of gains versus losses led different authors to sometimes make opposite conclusions based on the same information..

Recently, some possible scenarios by which regeneration may be lost have been discussed by Bely (2010) who argued that evolutionary loss of regeneration theoretically could occur by a single mutation that completely abrogates this ability, or gradually with increasingly restricted regenerative abilities. The present study showed that regeneration process in *M. infundibulum* is characterized not only by a slow rate of regeneration, a high mortality of body fragments (including 100% mortality of mid-pieces), as reported for *B. luctuosum* (Licciano et al. 2012), but also by the failure of thoracic segment regeneration. This could suggest the possibility that *M. infundibulum* is on an evolutionary trajectory toward losing regenerative abilities. At present, however, since phylogenetic relationships of Sabellidae are still not fully understood (although several hypotheses have been proposed: Fitzhugh 1989, 1991; Rouse & Fauchald 1997; Brown et al. 1999; Colgan et al. 2006; Rousset et al. 2007; Struck et al. 2007; Kupriyanova & Rouse 2008; Zrzavý et al. 2009; Capa et al. 2011), we cannot make strong inferences about the polarity of change in regenerative abilities in *M. infundibulum*. Moreover, as stated above, non-regenerating species should not necessarily be assumed to represent losses, and gain of regeneration is a possible reconstruction as well. A robust phylogenetic framework for interpreting data is required in order to confidently reconstruct the direction of evolutionary change.

Our findings based on the regenerative potential of Mediterranean specimens of *M. infundibulum* stand in contrast to the earlier findings of Wells (1952), which refer to specimens collected from the English Channel (Plymouth). In order to explain these differences, two alternative hypotheses may be suggested. (1) Assuming that specimens studied by Wells from Plymouth and those investigated in the present study from the Adriatic Sea are individuals of the same species, their different regenerative abilities may be due to the different cutting treatments performed in the two studies. The specimens studied by Wells (1952) were cut at just only one level along the body axis as the experiments performed by this author in fact aimed to investigate the role of the branchial crown in sabellid respiration. Bely (2006) reported that many species are suspected of being incapable of regenerating anteriorly or posteriorly based on the finding that they fail to regenerate following cuts at just one or a few axial positions, therefore the failure to regenerate does not necessarily indicate “absence”, but rather requires further studies under different experimental conditions. In this scenario, our findings showed that when worms are subjected to a cutting treatment different from that performed by Wells (1952), they can regenerate a complete posterior end and a partial (limited to the branchial crown) anterior end. (2) Another possible explanation for differences in regenerative abilities of Atlantic and Mediterranean specimens of *M. infundibulum* could be that the specimens studied by Wells (from Plymouth) and those investigated in the present study (from the Adriatic Sea) are in fact different taxa, or there is

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clinal variation in regenerative ability. Although 20 species have been included in *Myxicola* (Hartman 1959), only seven are considered valid. Most others have been synonymized with *M. infundibulum*, which was originally described from the Adriatic Sea by Renier (1804) and is at present the most commonly reported species in various localities around the world (Fauvel 1927; Day 1961; Imajima 1968; Goldman & Chandler 1986; Høisæter 1989; Langton & Robinson 1990; Hayward & Ryland 1995). As proposed by Dane (2008), it is likely that *M. infundibulum* actually represents a species complex whose members are very difficult to distinguish by focusing on only the traditional morphological characters. Past definitions of the genus *Myxicola*, for example, have regarded the nature of abdominal uncinigerous tori as important and have restricted attention only to the height of the palmate membrane, which is a rather dubious character on which to base the definition of the genus considering its variable development in species of other genera (Fitzhugh 1989). By contrast, Fitzhugh (1989) showed that the genus could be readily defined by a number of states not usually considered in the past, including the absence of ventral lips, a feature noted by Meyer already in 1888 but never used as a systematic character. Therefore, it is important to recognize the possibility that important features have been overlooked, and that more careful observations and analyses may allow taxonomic differentiation of Mediterranean and Atlantic specimens of *M. infundibulum*. A comparative study of specimens of *M. infundibulum* from the Mediterranean and specimens from Maine on the east coast of North America found that the European specimens were genetically and morphologically distinct, and were therefore likely to be different species (Dane 2008). Moreover, recently Giangrande et al. (2012) warned of the possibility that several species from worldwide localities are currently erroneously attributed to *M. infundibulum* and called for a revision of all Mediterranean material. It is therefore possible that specimens collected from Plymouth and identified as non-regenerating could actually be members of a different species than Adriatic *M. infundibulum*.

In conclusion, our results have revealed that *M. infundibulum* can no longer be considered a non-regenerating sabellid, providing experimental evidence that individuals of this species can in fact undergo regeneration of lost body parts, although to a lesser extent than other investigated sabellid species, including its congener *M. aethetica*. Before the present study, variation in regenerative abilities within sabellids had previously been described only for *B. luctuosum* and *S. spallanzanii*, which belong to closely related genera, and never reported between congeners. Given uncertainties regarding the phylogeny of Sabellidae and the paucity of available data on regeneration, we cannot currently confidently reconstruct the history of evolutionary transformations of regeneration in sabellids. Further analyses aimed at ascertaining the taxonomic status of individuals from worldwide localities are needed to verify if specimens collected from Plymouth and reported as non-regenerating are actually members of a different species than *M. infundibulum*. An integrated approach combining findings on regenerative ability of closely related taxa with developmental, evolutionary, ecological, and systematic data may ultimately help us explain the loss or retention of regeneration abilities within members of the genus *Myxicola* and the Sabellidae as a whole.

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ANSWER: "...or there is clinal variation" was added, without any other indication, by one of the reviewers. Here we are discussing about regeneration, thus I agree with you

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Figure legends

Fig. 1. Sampling site and the study species. **A.** Map of Italy showing the location of specimen collection (Brindisi Harbor). **B.** Adult specimens of *Myxicola infundibulum* acclimatized in aquarium prior to regeneration experiments.

Fig. 2. Experimental amputation treatments used in this study. **A.** 1-cut treatment. Each worm was cut into an anterior fragment (A1) which included the branchial crown, all the thoracic segments, and some abdominal segments, and a posterior fragment (P1) which included the remaining abdominal segments and pygidium. **B.** 2-cut treatment. Each worm was cut into an anterior fragment (A2) which included the branchial crown, all thoracic chaetigers, and some of the abdominal segments; a mid-body fragment (M) including only abdominal segments and with wounds at both anterior and posterior ends; and a posterior fragment (P2) which included the remaining abdominal segments and the pygidium.

Fig. 3. Survival of each type of body fragment and controls during regeneration experiments with individuals with and without gametes. **A.** Anterior body fragments from 1-cut treatment (A1); **B.** Posterior body fragments from 1-cut treatment (P1); **C.** Anterior body fragments from 2-cut treatment (A2). **D.** Posterior body fragments from 2-cut treatment (P2). **E.** Mid body fragments from 2-cut treatment (M). **F.** Uncut control worms.

Fig. 4. Early events of anterior end regeneration in posterior body fragments of *Myxicola infundibulum*. **A.** Wound healing at anterior end. The invagination of the cut surface due to constriction of surrounding tissues allows wound closure. **B.** Rudimentary branchial crown with outer superior margins crenulated (arrow) emerging from the invagination of the cut surface. **C.** Developing crown showing early branchial lobes (arrows). The anterior blastema (bl) formed after wound healing within the invagination of the cut surface is also clearly distinguishable. **D.** Branchial crown with 3 rounded radioles per lobe (arrows).

Fig. 5. Branchial crown regeneration in posterior body fragments of *Myxicola infundibulum*. **A.** Branchial crown with radioles (r) of differing length. **B.** Pigmented branchial crown with radioles increasing in number and length. **C.** Further developed branchial crown with anterior peristomial ring (apr) surrounding base of the structure. **D.** Anterior end of a posterior body fragment visible through the newly built tube (t).

Fig. 6. Posterior end regeneration in anterior body fragments of *Myxicola infundibulum*. **A.** Rudimentary anus (ra) emerging from invagination of cut surface. **B.** Posterior blastema (bl) formed after wound healing visible through newly built tube (t) of anterior body fragment. **C.** Formation of a small dark pigmented pygidium (py) from the posterior blastema (bl). **D.** Unpigmented abdominal chaetigers (ac) regenerated along growth zone added between pygidium (py) and original abdominal segments (ab).