Morphological investigations of posttraumatic regeneration in *Timarete* cf. *punctata* (Annelida: Cirratulidae)

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Abstract

Introduction: Annelids exhibit great regenerative abilities, which are mainly used after injury or during reproduction. These lophotrochozoans thus represent excellent models for regeneration research. However, detailed morphological studies concerning annelid musculature and nervous system redevelopment are limited to few taxa, and do not allow for broader comparisons and general conclusions regarding common patterns amongst annelids.

Results: Using immunohistochemical staining combined with confocal laser scanning microscopy (cLSM), we investigated the redevelopment of body wall musculature and nervous system during anterior and posterior posttraumatic regeneration in *Timarete* cf. *punctata*. Both regeneration processes start with wound healing, blastema formation, and blastema patterning. In posterior regeneration, this leads to the development of a new pygidium and a segment addition zone (SAZ) anterior to this structure. New segments are subsequently added in a sequential fashion. Anterior regeneration in contrast shows the formation of a new prostomium and peristomium first, followed by the simultaneous redevelopment of three segments, and an additional three segments in sequential order. Anterior muscular regeneration shows an outgrowth of longitudinal musculature from the residual body wall musculature, while circular musculature develops independently within the blastema. During posterior regeneration, new musculature becomes visible when the new segments reached a certain age. Neuronal regeneration begins with neurite outgrowth from the old ventral nerve cord in both cases, which are later forming loop structures. In anterior regeneration, the brain redevelops at the anteriormost position of the loops.

Conclusions: Posterior regeneration recapitulates normal growth from a certain timepoint with serial segment development by a posterior segment addition zone. Anterior regeneration is more complex, showing similarities to larval development in matters of the order, in which prostomium, peristomium, and segments are generated. Furthermore, we demonstrate the usefulness of regeneration studies to investigate morphological structures and evolutionary processes.

Keywords: cLSM, Musculature, Nervous system, Polychaetes, Sedentaria
In Cirratulidae, many members are known to reproduce by architomy, the separation of body fragments prior to the regeneration of anterior and/or posterior body end [11–13]. This mode is also found in the genus Timarete, as known for T. punctata, a widely distributed cirratulid, which is thought to represent a complex of closely related species [12, 14–17]. In this study, we describe the process of anterior and posterior posttraumatic regeneration in a member of the T. punctata complex. We investigated the redevelopement of musculature and nervous system by using immunohistochemical staining techniques combined with subsequent confocal laser scanning microscopy (cLSM). We further describe and discuss the adult myo- and neuroanatomy in comparison with those in other taxa, and report external morphological changes during regeneration. Finally, based on these new observations, we highlight general patterns of regeneration in annelids.

Materials and methods
Origin of investigated specimens and workflow of regeneration experiments
Specimens identified as Timarete punctata (Fig. 1h, i) were discovered in a seawater aquarium at the University of Leipzig. As, according to Magalhães et al. [17] T. punctata (Grube, 1859) represents a species complex, we used a DNA barcoding approach to characterize our specimens by sequencing fragments of the CO1 and 16S (see [18] concerning barcoding approach). Sequences for the specimens used, a maximum likelihood based phylogenetic reconstruction using the GTR + Γ + I model of sequence evolution, as well as pairwise sequence distances are given in the Additional file 1: Supplementary Information and Additional file 2: Figure S1.

Prior to the experiments, specimens were separated in a 30 l aquarium containing artificial sea water at a temperature of 27 ± 1 °C, sandy sediment, and a circulation pump. Within less than one month, they showed an enormous increase in number through asexual reproduction by architomy (it should be noted, that one year before the experiments also specimens with eggs were observed).

For performing regeneration experiments, specimens having a length between 10 and 15 mm were anesthetized in artificial sea water for about 10 min. Afterwards they were dissected between the tenth and eleventh chaetiger using a hollow needle. The anterior and posterior body parts were separated in PS boxes (Rotilabo®-Frischhaltebox Gerda, 1000 ml, Carl Roth GmbH, Karlsruhe, Germany) containing artificial seawater at a temperature of 27 ± 1 °C and an air influx. To enable daily fixation and to avoid inhomogeneity, based for example on long retention time in MgCl₂ solution, this was done by two sets of experiments (Table 1, set 1 and 2). For fixation, specimens were anesthetized in 7 % MgCl₂ dissolved in artificial sea water and subsequently fixed in a solution of 4 % paraformaldehyde in 0.1 M PBS (phosphate buffer solution, pH 7.4) overnight at 4 °C. After several rinses in 0.1 M PBS for at least 3 h at RT (room temperature), specimens were stored in PBS-azide (0.1 M PBS containing 0.02 % NaNO₃) at 4 °C until usage. Because first cLSM analyses revealed that fixation every day was not enough to illuminate all aspects of nervous system regeneration, a third set of experiments was performed to enhance the resolution of events (Table 1, set 3).

Immunohistochemistry
Anatomical details of body musculature and nervous system were investigated using standard immunohistochemical staining protocols and a range of well-established antisera. Standard negative controls were performed for all antisera, and in all cases the omission of the primary and/or secondary antiserum resulted in no staining. For analyses of the musculature, f-actin (=filamentous muscular actin) was stained with phalloidin-rhodamine and, for nervous system staining, antibodies against acetylated α-tubulin (structural component of microtubules, amongst others present in axons), FMRFamide (= small neuropeptide), and serotonin (=5-HT, neurotransmitter) were used.

Fixed whole mount animals were permeabilized in PBSTplus (0.1 mol l⁻¹ PBS containing 0.1 % NaNO₃ and 2 % Triton X-100) for 1 h and blocked in PBST-NGS (0.1 mol l⁻¹ PBS containing 0.1 % NaNO₃ and 0.1 % Triton X-100 with 6 % normal goat serum) overnight at RT. This step was followed by incubation in the primary antibody solution, either anti-FMFRAEamide (polyclonal antiserum raised in rabbit against fish and mammalian FMRFamide, supplied from Incstar, Stillwater, MN, USA, obtained via Acris Antibodies GmbH, Herford, Germany; dilution 1:500 in PBST-NGS), or a mixture of anti-acetylated α-tubulin (monoclonal anti-tubulin, acetylated antibody, produced in mouse, ascites fluid, Sigma-Aldrich, St. Louis, MO, USA; dilution 1:500 in PBST-NGS) and anti-serotonin (5-HT (Serotonin) rabbit antibody, lyophilized whole serum, Immunostar/Acris antibodies, Herford, Germany; dilution 1:500 in PBST-NGS) for 3 d at RT. After several rinses in PBST for about 4 h and PBST-NGS for 2 h, specimens were incubated in the secondary antibody solution, either only Alexa Fluor® 488 goat anti-rabbit IgG (H+L) (Invitrogen/Life Technologies, Darmstadt, Germany; dilution 1:500 in PBST-NGS) or a combination of this secondary antibody with Alexa Fluor® 568 goat anti-mouse IgG (H+L) (Invitrogen/Life Technologies, Darmstadt, Germany, dilution 1:500 in PBST-NGS) for 2 d at RT. Subsequently, specimens were rinsed in 0.1 mol l⁻¹ PBS for at least 4.5 h (combined anti-acetylated α-tubulin/anti-serotonin staining), or
Fig. 1 (See legend on next page.)
3 h (anti-FMRFamide staining) followed by incubation in a solution containing rhodamine-labelled phalloidin (Invitrogen, Carlsbad, CA, USA; 5 μl methanolic stock solution in 500 μl 0.1 mol 1−1 PBS) overnight for additional anti-f-actin staining. Subsequently, specimens were dehydrated in an ascending isopropanol series, treated in Murray's clearing solution (benzyl alcohol plus benzyl benzoate, 1:2), and mounted in DPX (dibutyl phthalate xylene, Sigma-Aldrich, St. Louis, MO, USA) between to cover slips.

Confocal microscopy and image processing
Specimens were analyzed with a confocal laser scanning microscope (Leica TCS STED, Leica Microsystems, Wetzlar, Germany). Confocal image stacks were processed with Leica LAS AF v2.3.5. and Leica LAS AF lite 3.3.10134 (both Leica Microsystems), as well as Fiji [19]. Drawings and final panels were designed using Adobe Photoshop CS6 and Illustrator CS6 (San Jose, CA, USA).

Scanning electron microscopy (SEM)
Specimens for SEM were anaesthetized in 7 % MgCl₂ solution (picric acid saturated aqueous stock solution, 37 % aqueous formaldehyde, and glacial acetic acid, 15:5:1) and dehydrated in an increasing ethanol series, ending with three changes of absolute ethanol. This was followed by critical point drying (CPG 030, BAL-TEC Union Ltd., LI) and sputtering with gold (Sputter Coater E5100 Series II ‘Cool’, Polaron Equipment Ltd., Watford, GB). SEM was performed using a Leitz 1000A (Wetzlar, DE). Drawings and final image plates were compiled as described above.

Results
Morphology of musculature and nervous system of adult specimens
The nervous system of adult specimens consists of a prominent anterior brain and a ventral nerve cord with distinct segmental nerves (Figs. 1a; 4a-b). The brain is situated in the prostomium and orientated towards anterior (Fig. 1a, b). While the brain always appears to be a compact structure on anti-serotonin and anti-acetylated-α-tubulin staining (Figs. 3j; 4a, b), anti-FMRFamide staining revealed a bipartite organization (Fig. 1a). Furthermore, in this staining there is a circular area without immunoreactivity in median posterior position (Fig. 1a, arrow). The brain is linked to the ventral nerve cord via two circumesophageal connectives (Fig. 1a). Each circumesophageal connective is composed of several neurite bundles, organized in a dorsal and a ventral root. Due to their fusion, an

Table 1 Overview of regeneration experiments. Time shift between experiments was three days for sets 1 and 2, as well as eight hours for set 3. At each time point, given number of anterior and posterior regenerating specimens was fixed. The total number is a summation of all specimens fixed in the particular experiments. Please note, that due to the dissection, this number is half of the number of specimens fixed. Additional specimens listed in brackets were dissected in reserve and placed back in a stock aquarium after fixation of the last day or hour.

| Set | Experiment | Start date | Fixation [days after dissection] | Specimens fixed in each case | Total number of specimens |
|-----|------------|------------|---------------------------------|-----------------------------|--------------------------|
| 1   | 1a         | 13.08.2013 | 2, 6, 8, 14, 16, 20, 22, 28, 30 | 5 anterior/5 posterior       | 45 (+10)                 |
|     | 1b         | 16.08.2013 | 4, 10, 12, 18, 24, 26           | 5 anterior/5 posterior       | 30 (+10)                 |
| 2   | 2a         | 21.08.2013 | 1, 5, 7, 13, 15, 19, 21         | 5 anterior/5 posterior       | 35 (+10)                 |
|     | 2b         | 24.08.2013 | 3, 9, 11, 17                     | 5 anterior/5 posterior       | 20 (+10)                 |
|     |            |            | Fixation [hours after dissection]|                             |                          |
| 3   | 3a         | 11.04.2014 | 52, 56, 60, 76, 80, 84           | 3 anterior/3 posterior       | 18 (+3)                  |
|     | 3b         | 11.04.2014 | 48, 64, 68, 72, 88, 92, 96       | 3 anterior/3 posterior       | 21 (+3)                  |
assignment of the particular neurite bundles to the roots is complicated in adult specimens, but possible based on their redevelopment during regeneration (see below). The ventral nerve cord is divided into two main strands, each of which is composed of several neurite bundles (Figs. 1a; 4a, b), but their exact number cannot be determined. One pair of ganglia can be found in each chaetiger, as well as in the peristomium. The ganglia of one segment almost contact at the midline of the body, thus making it impossible to elucidate the number of commissures connecting these ganglia (Fig. 3h, g). Three segmental nerves per ganglion run towards lateral. Whereas the first and third nerve is often stained weakly, the second segmental nerve, which extends into the parapodia, shows a more pronounced staining (Figs. 1a; 3f, h; 4b).

The body wall musculature of *T. cf. punctata* is composed of an outer circular and an inner longitudinal layer (Figs. 1c-f; 4c-h). The circular musculature is most prominent at level with the chaetae, but present throughout the entire body (Figs. 1c, d, f; 4d-h). The longitudinal layer is composed of four main strands, two of them located dorsal and two ventral. The dorsal longitudinal muscle strands form a plate, encircling the dorsal half of the body in more anterior segments and up to two thirds in posterior segments, with a border directly above the notochaetae (Fig. 1e, f, i). The ventral longitudinal muscle strands are more compact, reaching to the notochaetae and are penetrated by the neurochaetae, thus appearing partially bipartite (Fig. 1c, e; i). On its course to the posterior end, the longitudinal body wall musculature gets weaker and fuses inside the pygidium (Fig. 1d). At the anterior end, the longitudinal muscle strands fan out in the prostomium (Fig. 1c, i). There is an additional longitudinal muscle fiber in ventro-median position (Figs. 1e; 4d, e), which runs over the whole length of the body. Another bundle of muscle fibers with dorso-ventral orientation is running through the posterior part of the brain (Fig. 1a, c, arrow). Further musculature can be found associated with the parapodia, comprising those of the aciculae and chaetae, as well as a meshwork-like one around the intestine (Fig. 1e, f). Additionally, the mouth opening is supported by a large muscular pouch on its posterior side (Fig. 1c).

**Regeneration progress**

The survival rate of dissected specimens was up to 100 % in the different experimental settings. However, regeneration rates differed markedly between specimens. Moreover, specimens of the third set (Table 1) appeared to regenerate slower. To ensure the comparability of the presented data, we categorized regenerative stages (Fig. 2) according to external and internal morphological characteristics: Anterior regeneration is scaled in an invagination (ai), three blastema (ab1-3), two blastema patterning (ap1-2), a re-segmentation (ar), and a growth (ag) stage. Posterior regeneration comprises an invagination (pi), two blastema (pb1-2), a blastema patterning (pp) and a growth (pg) stage. The time specifications given below are approximated.

**Anterior regeneration**

Following dissection, the musculature contracted to close the wound, and thus caused an invagination at the cutting side (Fig. 2, ai). One day after dissection (dad), all investigated specimens remained in this stage. The second day, first indications of a developing blastema (Fig. 2, ab1) became visible. The third and fourth day after dissection were characterized by elongation of the blastema (Fig. 2, ap1-2). This was followed by a redevelopment of the mouth opening at 4–5 dad (Fig. 2, ap1) and the occurrence of the first pair of tentacles by 6–8 dad (Fig. 2, ap1-2). In all investigated specimens this pair redeveloped at the position of the later chaetiger three. During the late blastema patterning stage, also the boundary of prostomium and peristomium became slightly visible. The re-segmentation started with the first three segments at once by 6–11 dad (Fig. 2, ar). Afterwards, three more segments redeveloped sequentially (Figs. 3k, l; 4a, b). The maximum number of anteriorly regenerated segments was six and was redeveloped earliest at 11 dad. The redevelopment of further tentacles and the redevelopment of branchiae did not appear to cluster around any specific time point and, especially for the branchiae, no obvious pattern was visible. A second pair of tentacles occurred first in a specimen of 9 dad, this time at chaetiger 4. First branchiae were found at 7 dad. When all six segments were established, they grew to their final size (Fig. 2, ag). After 30 dad, most new anterior ends were nearly of same size, color and pigmentation compared to the residual segments. Nevertheless, they had fewer tentacles and branchiae than undissected specimens.

**Posterior regeneration**

Invagination (0–2 dad) and blastema formation (3–5 dad) were comparable to anterior regeneration (Fig. 2, pi and pb1–2). The first signs of blastema patterning was the formation of the anus at 4–6 dad (Fig. 2, pp). Afterwards, new segments were added successively always directly in front of the pygidium, so that the oldest newly developed segments were in the anteriormost position (Fig. 2, pg). About one week after dissection, the number of new segments was five to eight, and after two weeks up to 22. The first branchiae were observed at 20 dad in a specimen with 25 new segments. Likewise to anterior regeneration, there was no fixed pattern of branchiae occurrence recognizable.
Fig. 2 Schematic drawings of anterior and posterior regenerative stages in *Timarete cf. punctata*. The anus is colored in orange, branchiae in blue, chaetae in grey, mouth opening in yellow, nuchal organ in green, and tentacles in red. The dotted line in the upper drawing indicates the side of dissection. Please note that anterior and posterior stages show differences in characteristics and period. Posterior regeneration (left column) started with an invagination stage (pi, 0–2 days after dissection), followed by a blastema stage (pb1-2, 3–5 dad) with formation and development of a blastema. Afterwards, during the blastema patterning stage (pp, 4–6 dad) the anus became visible. During the growth stage (pg, from 6 dad onwards) new segments were added by a posterior segment addition zone (SAZ) directly anterior to the pygidium (py). The first stages of anterior regeneration with invagination (ai, 0–1 dad) and blastema formation (ab1-3, 2–4 dad) are comparable to the posterior regeneration, according to the outer morphology. In the early blastema patterning stage (ap1, 4–5 dad) the mouth opening redeveloped. Afterwards, the first pair of tentacles occurred (6–8 dad) and the boundaries of pro- (pr) and peristomium (pe) as well as the nuchal organs became visible by the late blastema patterning stage (ap2, 6–10 dad). With the formation of three segments at once, the re-segmentation stage (ar, 6–11 dad) was reached and continued with sequential addition of three more segments. Also branchiae and a second pair of tentacles were seen first at this stage. Finally, during the growth stage (ag, from day 11 onwards), all described structure increased in size until they reached an adult condition.
Fig. 3 (See legend on next page.)
Finally, during the fourth week after dissection, the transition from the first ten (residual) to the new developed (re-generated) segments became increasingly indistinct and specimens were no longer distinguishable from untreated ones.

**Anterior neuronal and muscular regeneration**

In the early blastema stage, no neuronal regeneration was detectable (Fig. 3a, b). With growth of the blastema, an infiltration of neurites into the blastema occurred. Within less than 3 days, a plexus had developed during the middle blastema stage (Fig. 3c, d). These neurites originated in the ventral nerve cord of the residual body. Also first signs of organization by fusion of neurites to bundles occurred at this stage, at least as visible in the anti-serotonin staining. By the end of 4 days, a state exhibiting several neuronal loops was reached within the late blastema stage (Fig. 3e–h). These loops included one median and two lateral loops. The neurites of the median loop were connected with the inner neurites of each strand of the ventral nerve cord (Fig. 3e, f), whereas each lateral loop was connected exclusively with the outer ipsilateral neurites of the ventral nerve cord. Depending on the specimen, the loops were orientated more anterior (Fig. 3e, f) or more dorsal (Fig. 3g, h), and showed different levels of mergence. During the end of the first week after dissection, the loops were stretched anterior according to blastema elongation and started to fuse (Fig. 3i). At the end of blastema patterning, the nerve loops were no longer distinguishable, as they were completely fused to the circumesophageal connectives (Fig. 3j). An assignment of the neurites respectively to the dorsal and ventral roots of the circumesophageal connective was impossible in some cases, but the outer ones which in particular showed a more intense staining referred to the dorsal root, whereas the inner ones represented the ventral root. Furthermore, the brain had redeveloped at the most anterior end of the regenerated nervous system and the nuchal organs became visible due to their ciliation. During the second week after dissection, the reformation of segments was accompanied by the redevelopement of the segmental nerves (Fig. 3k, l). As in adult specimens, the second segmental nerve was already the most prominent one.

On reaching the growth stage, the regeneration of the nervous system in the redeveloped anterior body end was complete (Fig. 4a, b).

The first signs of redeveloping body wall musculature occurred in the early blastema patterning stage in the middle of the first week after dissection (Fig. 4c–e). At this time point, thin longitudinal muscle fibers with an hourglass-like shape, first circular muscle fibers with origin in the lateral blastema, and a thin muscle ring surrounding the mouth opening became visible (Fig. 4e). Once started, the redevelopment of the musculature continued and during the late blastema patterning stage the circular layer became prominent (Fig. 4f). Furthermore, the musculature of the mouth opening started to redevelop the muscular pouch. With the early resegmentation in the second week after dissection the longitudinal musculature was well redeveloped and the ventral longitudinal muscle strands departed from each other to form their final shape (Fig. 4g). The development of the muscular pouch of the mouth opening also continued. When all regenerated segments were present in the growth stage by two weeks after dissection, all components of the body wall musculature as well as those of the mouth opening possessed their final shape (Fig. 4h).

**Posterior neuronal and muscular regeneration**

Posterior nervous system regeneration started with an ingrowth of neurite bundles into the blastema in the early blastema stage (Fig. 5a–c) at the third day after dissection. While in most specimens the first neurite bundles originated in the inner neurites of the ventral nerve cord (Fig. 5c), in some specimens these originated in the outer ones (Fig. 5b). During the late blastema stage at
Fig. 4 (See legend on next page.)
four days after dissection the neurite bundles had grown and originated in all parts of the residual ventral nerve cord (Fig. 5d, e). In comparison with the anti-acetylated α-tubulin staining (Fig. 5d), the anti-serotonin staining (Fig. 5e) visualized more compact bundles. Furthermore, they were clearly assignable to the inner and outer parts of the ventral nerve cord. The inner neurite bundles connected at the most posterior position, thus forming a terminal loop. When regeneration continued, the neurite bundles fused and formed the final ventral nerve cord (Fig. 5f). With the addition of subsequent segments, the posterior nervous system showed its adult shape (Fig. 5g).

The redevelopment of the body wall musculature is described only briefly, as in most cases the staining did not allow the resolution of all details. During the blastema stages as well as the blastema patterning stage, no muscle fibers were detectable inside the regenerating posterior body part (Fig. 5h, i). The longitudinal muscle strands and circular fibers first became visible in redeveloped segments (Fig. 5j). In older regenerated segments, both layers were well defined, but the younger the segments the more indistinct the musculature was, especially in the circular one. In the youngest regenerated segments no muscular elements were detectable.

**Discussion**

**Adult morphology**

The basic architecture of body wall musculature in *Timarete cf. punctata* is comparable to that in other cirratulids [20, 21]. Compared with *T. anchylochaeta* [21] (named as *Audouinia anchylochaeta* Schmarda, 1861; source of synonymy: [22, 23]), the ventral longitudinal muscle strands are of comparable shape and extent in the anterior body. Differences occur in the dorsal longitudinal muscle strands, which cover only one third of the body in *T. anchylochaeta*. In comparison with *Cirratulus cf. cirratus* [20], there are some differences in the shape of longitudinal muscle strands; the dorsal longitudinal musculature of *C. cf. cirratus* is completely fused to a plate and the ventral longitudinal muscle strands are more compact. Future studies are necessary to understand if the differences between these cirratulids are reflected by the phylogeny.

Longitudinal muscle layers composed of four main strands have been identified in several annelid families and this feature is thought to represent a part of the hypothetical myoanatomical ground pattern [24–27]. A ventral longitudinal muscle fiber also exists in other annelid families [28–31]. As has been shown in numerous investigations, a well-developed circular musculature is typical for burrowing annelids [26], a lifestyle also typical for our investigated species. The muscular pouch of the mouth opening is part of the ventral pharyngeal organ, a structure already described in cirratulids [20, 32].

A dorso-ventral muscle bundle penetrating the brain of *T. cf. punctata* is not detectable in *C. cf. cirratus* [20]. However, distinct muscle fibers with similar orientation are described for *Ctenodrilus serratus* [33]. In this species, these fibers divide the brain in an equally sized anterior and posterior neuropil. Contrary, in *T. cf. punctata* the penetration of muscle fibers lies in between the posterior commissures or between the brain and a nerve directly behind it, which cannot be finally clarified. However, muscles extending in a dorso-ventral direction through the prostomium are also described in other Cirratuliformia, such as *Cossura pygodaclata* [28].

Apart from this, the nervous system of *T. cf. punctata* is largely comparable to that of *C. cf. cirratus* [20], as expected due to the close phylogenetic relationship (Additional file 2: Figure S1). Both possess a tetraneuralian ventral nerve cord with three segmental nerves leaving each ganglion laterally.

The brain is orientated anteriorly and the circumesophageal connectives are composed of two roots each. However, in adult specimens of *T. cf. punctata* the dorsal and ventral roots of the circumesophageal connective as well as the paramedian and main connectives of the ventral nerve cord are fused, so that distinguishing between
these is only possible based on their redevelopment during regeneration. This underlines the importance of developmental studies to investigate nervous system architecture, as already suggested by Müller [34]. Based on her hypothesis, annelids possess a pentaneuralian ventral nerve cord in the ground pattern. Comparing our findings with the phylogeny, Cirratulidae are part of the Cirratuliformia, which comprise amongst others also the Flabelligeridae, and are the sister taxon of the Siboglinidae [10, 35]. Together with the Orbiniida they constitute a sister clade to the other Sedentaria [35]. Neither in the flabelligerid Poeobius meseres [36] nor in the siboglinid Lamellibrachia satsuma [37] the median connective is described, but it is present in the orbiniids Proscopolas cygnocetus and larvae of Scoloplos armiger [38, 39]. Thus, the absence of a median connective in the ventral nerve cord might represent an autapomorphy of the Cirratuliformia/Siboglinidae clade. The existence of three segmental nerves represents the typical condition in Cirratulidae and reflects the supposed ancestral state in annelids [40, 41]. Typically, the nerve that innervates the parapodial appendages is the thickest [39]. Although parapodial cirri are absent in cirratulids, the most prominent segmental nerve is also the second one, which runs to the parapodia, so that the innervations of further parapodial structures can be assumed.

Regeneration process

Cirratulids are known for their extensive regeneration capability [20, 42, 43], which is also frequently used during asexual reproduction [12, 15]. Compared with the detailed descriptions of anterior regeneration in Cirrineris sp. [42] and Cirratus cf. cirratus [20], Timarote cf. punctata shows an average speed of regeneration. While in the latter the maximum number (see below) of regenerated anterior segments is first reached after 11 days, it takes eight in Cirrineris sp., but 14 in C. cf. cirratus. Interestingly, a blastema is visible in T. cf. punctata on the second day after dissection, but in Cirrineris sp. the first signs of a blastema are visible not earlier than by the third day. This suggests that anterior regeneration is initiated earlier in T. cf. punctata, but needs longer for full redevelopment of all structures. Furthermore, the tentacles were redeveloped in C. cf. cirratus and Cirrineris sp. at the same time with the start of re-segmentation, but develop prior to re-segmentation in T. cf. punctata. All three cirratulids show a nearly constant number of regenerated anterior segments, which is always less than removed. It appears that they have a kind of minimal functional unit composed of prostomium, peristomium and a limited number of chaetigers (six to seven in Cirrineris sp., five in C. cf. cirratus, six in T. cf. punctata) which is exclusively regenerated. This observation is in line with investigations in different annelid taxa [43–45]. However, in others the number of anterior regenerated segments usually depends on the cutting side [46–48].

Notably, the sequence in which the anterior segments were regenerated differs between the investigated cirratulids: While C. cf. cirratus always regenerates all anterior segments at once, Cirrineris sp. first regenerates four and T. cf. punctata only three segments and the remaining ones were regenerated subsequently [20, 42]. This might imply the existence of an anterior growth zone as supposed for several syllid species [29, 49, 50]. Furthermore, this contradicts the conclusion of Balavoine [3], that sequential addition of segments is absent during annelid anterior regeneration. Given that T. cf. punctata starts with three segments, there is a striking similarity to the ontogeny, where the larvae develops the first three chaetigers at once and all others are generated subsequently by the (posterior) segment addition zone between the last chaetiger and the pygidium [9]. Transferring this to T. cf. punctata, a reactivation of a larval developmental program might explain the findings. However, there is no support for such a reinitiation of larval developmental patterns during regeneration in Cirrineris sp. and C. cf. cirratus [20, 42]. An alternative explanation is that...
the remaining segments are already determined but poorly developed.

Another interesting point is the order of tentacle redevelopment. Based on the occurrence of tentacles, cirratulid genera can be subdivided in three groups: (1) without tentacles (former Ctenodrilidae), (2) one pair of tentacles (bitentaculate; e.g., Aphelochaeta, Chaetozone, Dodecaceria), or (3) two or more groups of tentacle filaments (multitentaculate; e.g., Cirratus, Cirriformia, Timarete) [51, 52]. The absence of tentacles was shown to be a derived character e.g., [53], but it is unknown, if the bitentaculate or the multitentaculate genera represent the ancestral stage. Based on our findings, we hypothesize that the occurrence of more than one pair of tentacles is the derived character: In T. cf. punctata, one pair is regenerated first on chaetiger three (later groups one and two) and the second one then follows on chaetiger four (later groups three and four). This implies that this first pair on chaetiger four represents a duplication of tentacles, otherwise simultaneous development may be accepted. Subsequently, the number of tentacles increases in all four groups. Interestingly, in other multitentaculate cirratulids the branchiae are developed first [54, 55] or together with the first tentacles [56] during ontogeny.

During posterior regeneration, the pygidium and the posterior segment addition zone (SAZ) are redeveloped. This is followed by segment addition, which is comparable to normal growth, as supposed by Balavoine [3] or Gazave et al. [57]. Given that this process of posterior regeneration is widespread in annelids [29, 50, 58, 59], it presumably represents a plesiomorphic condition. Nevertheless, Moment [60] reported a nearly simultaneous formation of all regenerated segments in the maldanids Clymenella torquata and Axiothella mucosa, while A. rubrocincta again showed serial segment addition. This exception may be due to the constant number of segments present in most maldanids [61].

Finally, it is clear that regeneration speed varies between specimens within one set of experiments as well as between different sets. Because experimental parameters were kept constant, these differences should be based on individual characters: T. cf. punctata showed frequent architomy, thus specimens of same size could have very different ages and consequently different regeneration speed. Also nutritional conditions may be important. Differences between the first two sets and the third one could be based on the time shift and the potentially different situation of individuals within their respective reproductive cycle (e.g., change between growth and asexual reproduction) which can slow down regeneration due to limited resources [62–64].

Regeneration of musculature and nervous system

The regeneration of the body wall musculature during posterior regeneration mirrors the normal posterior growth [9, 65, 66]. During anterior regeneration, there are differences in the redevelopment of the circular and the longitudinal layer: While longitudinal musculature shows an outgrowth originated in its correspondent structures within the residual (old) body, the circular musculature develops independently inside the blastema. The same was found in other annelid species as well, which also show a slower regeneration of the musculature as compared to the nervous system [20, 67].

During anterior regeneration the first signs of the redeveloping nervous system are outgrowing nerve fibers with origin in the ventral nerve cord of the residual body (Fig. 6), which is comparable to other annelids [67–70]. This is followed by the development of a three-loop structure, as in Cirratus cf. cirratus [20]. However, there are differences in Timarete cf. punctata. First, the median loop is orientated anteriorly from the outset and, in addition, it is initiated in a compressed position. Later, this structure is stretched according to blastema elongation. Afterwards, all three loops fuse to form the circumesophageal connectives, in which the median loop becomes the ventral root and the lateral loops the dorsal root of the circumesophageal connectives. The occurrence of three closed loops is not described outside of cirratulids, but in Dorvillea bermudensis a related structure composed of a closed median loop connected to the inner neurites of the ventral nerve cord and two lateral neurite bundles connected each to the outer parts of the ventral nerve cord is reported [70]. Moreover, circumscribable roots of the circumesophageal connectives were also found in amphinomids, enchytraeids, naidids, or spionids [69, 71, 72]. Segmental nerves were redeveloped together with their segments, but ganglia are hardly visible at this stage. However, they must be present to interconnect the segmental nerves with the ventral nerve cord.

The nervous system redevelopment during posterior regeneration shares some major similarities with the anterior one. There is also an outgrowth originated in the residual ventral nerve cord and a loop-like structure occurs as well (Fig. 6). However, this structure solely refers to the inner neurite bundles. Later, the outer ones also fuse with this terminal loop. This terminal loop is presumably homolog to the terminal commissure found in D. bermudensis [70]. The main difference between anterior and posterior nervous system regeneration is the time point of reoccurrence for segmental nerves and ganglia: During anterior regeneration they redevelop together with the segments, while in posterior regeneration their redevelopment is delayed until the new segments have reached a certain age.
Conclusions
In this study we investigated the redevelopment of external structures, the musculature, and the nervous system during posttraumatic regeneration. Although early anterior and posterior regeneration with wound healing, blastema formation and patterning are largely comparable, both processes show remarkable differences in later redevelopment. While posterior regeneration appears to be a recapitulation of "normal" growth, anterior regeneration shows a unique pattern with similarities to the ontogeny of segment formation. Remarkably, the redevelopment of the nervous system during anterior and posterior regeneration is realized with loops connecting both main strands of the ventral nerve cord, which were later stretched. Given that related processes were found in other annelids, too, this might represent a plesiomorphic condition.
Furthermore, we demonstrate how regeneration studies can yield new insights into anatomical patterns, ontogeny, and evolutionary processes. Although well investigated in some model annelids, such as Capitella teleta or Platynereis dumerilii, data for most annelid taxa are lacking. In these annelids, especially in those for which breeding is impossible, regeneration studies represent a powerful tool to close the gaps in our knowledge.

**Additional files**

**Additional files 1: Supplementary information.** Additional material and methods for Supplementary Figure S1.

**Additional files 2: Figure S1.** Maximum likelihood tree based on 16S and CO1 sequences of available Timarete and Cinformia sequences.

**Abbreviations**

ab1-3: Blastema stage 1–3 of anterior regeneration; ag: Growth stage of anterior regeneration; an: Anus; ai: Regeneration stage of anterior regeneration; ar: Rear-regeneration stage of anterior regeneration; bn: Brain; bw: Brain; cc: Circumesophageal connective; cm: Circular musculature; ci: Cerebral ganglion; cl: Capsular loop; cm: Circular musculature; cs: Cerebral ganglion; dl: Dorsal longitudinal musculature; d1: Dorsal root of circumesophageal connective. 

d: Dorsal; d1: Dorsal root of circumesophageal connective; d2: Dorsal longitudinal connective; d3: Dorsal longitudinal connective; d4: Dorsal lateral connective; d5: Dorsal lateral connective. 

d1: Dorsal root of circumesophageal connective; d2: Dorsal longitudinal connective; d3: Dorsal longitudinal connective; d4: Dorsal lateral connective; d5: Dorsal lateral connective. 

d1: Dorsal root of circumesophageal connective; d2: Dorsal longitudinal connective; d3: Dorsal longitudinal connective; d4: Dorsal lateral connective; d5: Dorsal lateral connective. 

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contribution**

MW and CB designed the project. MW cultured the animals, did all experimental work, analyzed the data, drafted the manuscript and generated the figures. CH contributed to the interpretation of data. All authors read and improved the final manuscript.

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