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REGULAR ARTICLE

No head regeneration here: regeneration capacity and stem cell dynamics of *Theama mediterranea* (Polycladida, Platyhelminthes)

Philip Bertemes[1] · **Alexandra L. Grosbusch**[1] · **Bernhard Egger**[1]

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Abstract

Research on the regeneration potential of flatworms (Platyhelminthes) has been mainly undertaken with planarians (Tricladida), where most species can regenerate a head and no proliferation takes place in the blastema, i.e. the early undifferentiated regenerative tissue. Only few studies are available for an early-branching group within the Platyhelminthes, the Polycladida. Head regeneration in polyclads is not possible, with a single exception from a study performed more than 100 years ago: *Cestoplana* was reported to be able to regenerate a head if cut a short distance behind the brain. Here, we show that '*Cestoplana*' was misdetermined and most likely was the small interstitial polyclad *Theama mediterranea*. We revisited regeneration capacity and dynamics of *T. mediterranea* with live observations and stainings of musculature, nervous system, and proliferating and differentiating stem cells. In our experiments, after transversal amputation, only animals retaining more than half of the brain could fully restore the head including the brain. If completely removed, the brain was never found to regenerate to any extent. Different from planarians, but comparable to other free-living flatworms we detected cell proliferation within the posterior regeneration blastema in *T. mediterranea*. Similar to other free-living flatworms, proliferation did not occur within, but only outside, the differentiating organ primordia. Our results strongly imply that brain regeneration in the absence of the latter is not possible in any polyclad studied so far. Also, it appears that proliferation of stem cells within the regeneration blastema is a plesiomorphy in flatworms and that planarians are derived in this character.

Keywords Flatworm · Turbellarian · Planarian · Neoblast stem cells · Blastema

Introduction

Polyclads are an early branching, free-living, almost exclusively marine clade within the species-rich phylum Platyhelminthes (flatworms) (Egger et al. 2015; Laumer et al. 2015; Laumer and Giribet 2017). Adults are hermaphrodites and normally range between 1 and 15 cm in length with about 800 described species (Prudhoe 1985; Martín-Durán and Egger 2012). They have a simple body plan, are dorsoventrally flattened and derive their name from their highly ramified gut (Hyman 1951). Traditionally, polyclad systematics supports two large taxa: cotyleans, having a sucker posterior of the genital openings; and acotyleans without such a sucker (Lang 1884).

Theama mediterranea was described from the interstitial of the lower intertidal zone all around the Mediterranean Basin (Italy, Greece, Croatia, Israel, France, Tunisia) (Curini-Galletti et al. 2008; Gammoudi et al. 2017). The almost fully transparent adults are very elongated, reaching a length of about 20 mm and a width of 2.5 mm. They have two rows of a varying number of tentacular eyes and two pairs of cerebral eyes situated directly anterior to the brain (Curini-Galletti et al. 2008). The pharynx is located in the middle of the body, and an adhesive pad is located ventrally at the posterior tip of the animal (Fig. 1; see also Tyler (1976)).

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✉ Bernhard Egger
bernhard.egger@uibk.ac.at

Philip Bertemes
philip.bertemes@student.uibk.ac.at

Alexandra L. Grosbusch
alexandra.grosbusch@student.uibk.ac.at

[1] Institute of Zoology, University of Innsbruck, Technikerstrasse 25, 6020, Innsbruck, Austria

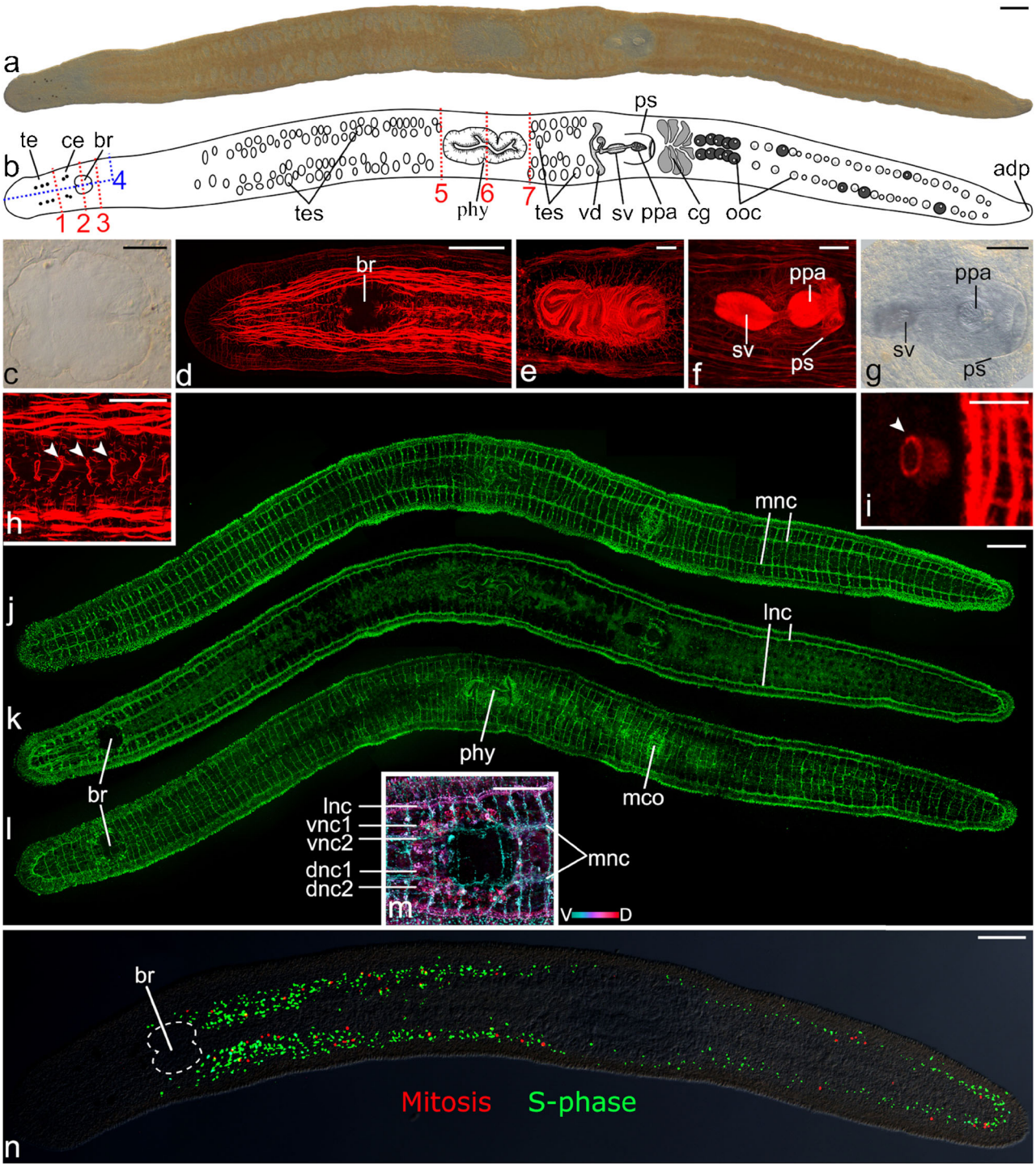

Fig. 1 Overview of a mature *Theama mediterranea*. **a** Differential interference contrast (DIC) of an adult *T. mediterranea*. **b** Schematic drawing of the animal above: two irregular rows of tentacular eyes (*te*), two pairs of cerebral eyes (*ce*), brain (*br*), two stripes of follicular testes (*tes*) in the anterior half of the animal, interrupted by a ventrally lying ruffled pharynx (*phy*), and followed by the genital apparatus with vasa deferentia (*vd*), seminal vesicle (*sv*), penis papilla (*ppa*), penis sheath (*ps*), cement glands (*cg*). The oocytes (*ooc*) lie in two stripes ranging between cement glands and the tip of the tail, the latter having a ventrally lying adhesive pad (*adp*). Dotted lines indicate the amputation levels (1–7). **c**–**i** DIC images and confocal projections of F-actin musculature of the brain (**c**, **d**), the ruffled pharynx (**e**) and the male copulatory organ (**f**, **g**). **h** Circular muscles surrounding the intestine. **i** Conical-shaped, phalloidin-positive structure in the epidermis. **j**–**l** Confocal projections of the AVRLIRLamidergic nervous system. Ventral (**j**), central (**k**) and dorsal (**l**) projections. **m** Temporal-colour coded image of the head including the brain (blue is more ventral, red is more dorsal) with main nerve cords (mnc), lateral nerve cords (lnc) and two pairs of ventral (vnc1-2) and dorsal nerve cords (dnc1-2). **n** Staining of mitotic cells (H3, red) and S-phase cells (BrdU pulse, green) in a sub-adult specimen without copulatory organs. *Scale bars* a, j–l: 200 μm; d, m–n: 100 μm; c, e–h: 50 μm; i: 5 μm

Over 160 years ago, Dalyell was the first to report regeneration phenomena in polyclads (Dalyell 1853). The first dedicated work on polyclad regeneration was published more than a century ago (Monti 1900), but only two studies dealing with polyclad regeneration were published within the last 20 years (Lapraz et al. 2013; Okano et al. 2015). In a nutshell, in most cotylean and acotylean polyclads, the ability to regenerate all parts posterior to the brain was observed. However, several polyclad species were reported to regenerate only small, peripheral body parts (Monti 1900; von Levetzow 1939). Many studies stressed the importance of the brain for complete head regeneration, i.e. the brain was not regenerated if completely removed. In contrast to the anterior and lateral halves, the posterior half of the brain was never found to be regenerated (Child 1904a; 1904b). All other organs, such as eyes and pharynx, could be regenerated in some species, even in the absence of the brain (e.g. von Levetzow 1939).

In a polyclad originally identified as '*Cestoplana*', one important difference to all other studied polyclad species was observed: the complete brain could be regenerated if the cut was made just behind the brain (Child 1905b). To date, these experiments from 1905 remain the only evidence of complete brain regeneration in polyclads, and when the so-called polyclad rule for regeneration was created 17 years later, it cautiously stated that polyclads 'are able to restore missing parts, provided the cephalic ganglia are intact' (Olmsted 1922).

While most studies on polyclad regeneration were focussed on determining the regeneration capacity, only two studies provided some insights into stem cell dynamics during regeneration (von Levetzow 1939; Okano et al. 2015). In flatworms, missing tissues are replenished by pluripotent stem cells, the so-called neoblasts (Wagner et al. 2011). They are the only proliferating cells in juveniles and adults, are responsible for tissue maintenance, growth, and regeneration (Baguñà et al. 1989), and are conspicuously lacking in the epidermis of rhabditophoran flatworms, including polyclads (Egger et al. 2009b; Lapraz et al. 2013; Okano et al. 2015). In triclads, proliferating cells were reported to be lacking also in the regeneration blastema (Saló and Baguñà 1984; Morita and Best 1984), although newer studies indicate that mitoses are occasionally found inside the blastema (Wenemoser and Reddien 2010). In other free-living flatworms, the regeneration blastema is a centre of proliferation (Egger et al. 2009a; Dirks et al. 2012; Girstmair et al. 2014).

With this study, we expand the available dataset on regeneration in flatworms, which is currently very limited for all flatworm orders except the triclads (Egger et al. 2007). We specifically address the following questions: can the complete brain regenerate in *Theama mediterranea*, or at least parts of it? To what extent can the head region anterior to the brain regenerate in the absence of the brain? Are there proliferating cells within the blastema, or only outside the blastema? Are there proliferating cells in the regenerating organ primordia? To answer these questions, we used live observations and stem cell, muscle, and nervous system fluorescence stainings of controls and regenerating adult animals.

Materials and methods

Sampling and animal cultures

Interstitial material containing *Theama mediterranea* was sampled at the water line in surficial coarse sand in July and September 2015, March 2016, and March and October 2017 in Rovinj, Croatia (45.1180406 N, 13.616976 E) and in October 2018 in Punat, Croatia (45.008536 N, 14.622129 E). The samples in seawater were mixed 1:2 with 7.14% $MgCl_2 \times 6\ H_2O$ (magnesium chloride hexahydrate) and incubated between 5 and 30 min at room temperature, mixed well and poured through a 60-μm mesh. Collected specimens were taxonomically determined by eye with a stereomicroscope. Animals were kept in groups of 20–30 individuals in Petri dishes in artificial seawater (ASW) with a salinity of 3.5% at 15 °C in darkness, with water changes every 2 to 4 weeks. They were fed with either freshly killed and chopped up *Palaemon varians* or with thawed and chopped up *Artemia salina* (both Crustacea). Animals were cultured for up to 7 months in the laboratory in Innsbruck, Austria.

S-phase labelling and fixation

Starved, intact animals were S-phase labelled twice in simultaneous pulse- and pulse-chase experiments. For the pulse-chase part of the experiments, animals were soaked for 60 min in 0.4 mM 5-ethynyl-2′-deoxyuridine (EdU, Invitrogen, USA) in ASW in darkness at room temperature (RT). Afterwards, they were rinsed two times in ASW and relaxed in 7.14% $MgCl_2 \times 6\ H_2O$ before amputation with a razor blade on a glass slide. Amputation sites are shown in Fig. 1. Both parts of the amputated animals were transferred to a Petri dish containing ASW and maintained without feeding to regenerate. After 7 or 14 days, the regenerates were soaked for 60 min in 5 mM 5-bromo-2′-deoxyuridine (BrdU, Sigma-Aldrich, USA) in darkness at RT for the pulse part of the experiments. The specimens were subsequently rinsed two times in ASW before each animal was individually relaxed in a droplet of 7.14% $MgCl_2 \times 6\ H_2O$ for several seconds (up to 1 min maximum) on a glass slide prior to fixation in 4% formaldehyde (made from paraformaldehyde, Sigma-Aldrich, USA) in 0.1 M

PBS (phosphate-buffered saline) for 60 min at RT. After this, the specimens were rinsed seven times with PBS-T_x (PBS with 0.1% Triton X-100 (Thermo Fisher Scientific, USA)) over a period of 60 min at RT.

Immunocytochemistry and fluorescent staining

F-Actin musculature and the amidergic and serotonergic nervous systems were visualised with phalloidin and antibodies against AVRLIRLamide (custom-made, see Lapraz et al. (2013)) and serotonin (5-hydroxytryptamine, 5-HT, DakoCytomation, Denmark), respectively. Fixed animals were incubated for 2 h in BSA-T_x (PBS-T_x with 1% bovine serum albumin, Carl Roth, Germany), followed by incubation in the first antibody, either rabbit-anti-AVRLIRLamide (1:50 in BSA-T_x) for 48 h at 4 °C, or rabbit-anti-5-HT (1:3000 in BSA-T_x) overnight at 4 °C. A washing step in PBS-T_x for 24 h at 4 °C was performed, with ten changes of washing solution in total. A second incubation in BSA-T_x for 1–2 h at RT followed, and then 60-min incubation in darkness at RT in the FITC-conjugated secondary antibody swine-anti-rabbit (1:250 in BSA-T_x, Dako, Denmark), together with the site-selective probe tetramethylrhodamine-conjugated phalloidin (1:250 in BSA-T_x, Sigma-Aldrich, USA). Several washing steps over a period of 72 h were done, changing PBS-T_x at least 10 times at 4 °C.

All fixed specimens containing BrdU and EdU (or only BrdU) labels were incubated in Protease XIV (Sigma, USA) for 15 min at 37 °C. After this, they were incubated for 60 min in 2 M HCl at 37 °C. Then, they were washed several times over a period of 60 min in PBS-T_x before being incubated another 60 min in BSA-T_x at RT. Specimens were incubated overnight at 4 °C in the primary antibody mouse-anti-BrdU (1:600 in BSA-T_x, Developmental Studies Hybridoma Bank, USA). Several PBS-T_x washing steps were performed over the next 24 h. A further 60-min BSA-T_x block was performed before adding the secondary antibody Alexa-555-conjugated goat-anti-mouse (1:250 in BSA-T_x, Life Technologies, USA) for 60 min in the dark at RT. After this, the secondary antibody was removed by washing several times with PBS-T_x for 60 min. This step concluded the BrdU staining. For EdU-only stainings, the BrdU staining was omitted. For EdU stainings, the specimens were incubated another 60 min in BSA-T_x at RT. The EdU reaction mix was freshly prepared according to manufacturer's instructions (Click-iT, Invitrogen, USA). Specimens were incubated for 2 h in the mixture. Next, the specimens were washed at least 10 times over 72 h with PBS-T_x at 4 °C.

Specimens used for calculating the ratio between S-phase neoblasts and mitotic cells were treated as above (BrdU and EdU), except a second primary antibody, rabbit-anti-H3 (Millipore, USA) and the secondary antibody Alexa-568-conjugated goat-anti-rabbit (Invitrogen, USA) were added. To remove the unbound secondary antibody, the specimens were washed several times overnight at 4 °C in PBS-T_x, rendering the animals ready to mount.

Specimens were mounted in VectaShield (Vector Labs, USA) on glass slides, sealed with nail polish and stored at −20 °C. The primary or the secondary antibody was omitted in AVRLIRLamide and 5-HT staining controls. In EdU and BrdU staining controls, the respective thymidine analogue was omitted.

Microscopy and visualisation

Live animals were observed, amputated, and handled under a Nikon SMZ645 and SMZ-2B stereomicroscope (Nikon, Zürich). Squeeze preparations of animals were photographically documented using a Leica DM5000 B microscope, equipped with either a Leica DFC 490 or a Leica DFC 495 camera (Leica, Germany). All confocal stacks were generated on a Leica TCS SP5 II confocal microscope. Figures and schematics were made in GIMP up to version 2.10 (https://www.gimp.org/), Fiji up to version 1.52i (Schindelin et al. 2012) and Inkscape up to version 0.92 (https://inkscape.org/). Images that show internal structures of the animal (e.g. the nervous system, inner musculature, stem cells) are partial Z projections. We omitted the ventralmost and dorsal-most planes from the confocal stack to remove signal from unspecific staining in the rhabdites. In all images, animals are facing up or left. Mitotic cells and S-phase neoblasts were counted in three non-overlapping 100 μm × 100 μm boxes in the respective body regions in total confocal projections.

Results

Control animals

General observations

Mature animals with a penis and oocytes were found between January and July (Fig. 1a). Only two cocoons (April 2016 and 2018, respectively) were found in the cultures during this study. A ciliated longitudinal groove was apparent on the ventral side of the rostrum. A patch of duo-gland adhesive organs was situated at the ventral side of the tip of the tail.

Adult animals were observed with a total eye number between 3 and 15 (n = 119 animals). Typically six to eight tentacular eyes (three to four in each row) were found, but specimens with zero or even five tentacular eyes in a row were observed. Two pairs of cerebral eyes lay slightly anterior to the brain, with a large majority of animals

featuring three to four cerebral eyes in total. Only 2.5% of the observed animals had less than three cerebral eyes, and 5.0% had more than four (up to six) cerebral eyes (see Fig. S1 for variations of eye numbers).

F-Actin musculature

Longitudinal muscle fibres were the most prominent fibres in the body wall with a width of 1.2 ± 0.3 μm (n = 30 fibres). Less prominent were the circular (innermost) and diagonal (in between) muscle fibres with a width of only 0.8 ± 0.2 μm (n = 30 fibres). The ventral view revealed three openings, which are (from anterior to posterior) the mouth opening, the male pore, and the female pore. The encapsulated brain was enclosed by a distinct meshwork of circular muscles and a few longitudinal muscles; conspicuous swirls of muscles surrounded the capsule. The region dorsal and ventral to the brain completely lacked dorso-ventral muscles. No muscle fibres were detected within the brain capsule (Fig. 1d). The muscle-free region ranged 95.8 ± 9.0 μm in length and 85.1 ± 4.2 μm in width (n = 10 specimens). The musculature of the ruffled pharynx consisted of a dense meshwork of longitudinal and circular fibres (Fig. 1e). The most complex muscular structure in an adult *T. mediterranea* was the male copulatory organ (Fig. 1f, g). The penis sheath, surrounding the penis papilla, showed several thin longitudinal muscle fibres forming a hollow ellipsoid with a wide opening at the posterior end (Fig. 1f). The penis papilla was formed by thin criss-crossing diagonal muscles, thus forming a fine meshwork. Circular muscles, apparently surrounding the main branch of the intestine, were equidistantly distributed (23.9 ± 6.1 μm, n = 20 fibres) in a single row throughout the whole animal (Fig. 1h, arrowheads). The epidermis housed several conical, phalloidin-positive structures randomly distributed on both sides of the animal (Fig. 1i).

Nervous system

A strictly orthogonal arrangement (see Reisinger 1925) of the AVRLIRLamidergic nervous system was found in *T. mediterranea*. It consisted of an anteriorly positioned brain, from which four pairs (2 ventral and 2 dorsal, vnc1-2 and dnc1-2, Fig. 1m) of longitudinal nerve cords (NCs) emerged to anterior, and a single pair of ventral NCs (the main NCs, Fig. 1m, mnc) emerged from the posterior part of the brain. The main NCs formed a loop in the caudal region of the animal. A pair of peripheral and lateral NCs looped anteriorly and posteriorly (Fig. 1m, lnc). All pairs of NCs were interconnected by equidistantly placed orthogonal commissures, 43.3 ± 6.3 μm (n = 20 fibres) apart from each other. The encapsulated brain conspicuously lacked signal in most staining attempts (but see Fig. S2) and was therefore distinguishable as an unstained region (Fig. 1k–m). Numerous AVRLIRLamide-positive clusters were visible around the brain, anterior more than posterior (Fig. 1k–m). Dorsal NCs were not observable in AVRLIRLamidergic staining (Fig. 1l). The pharynx and the male copulatory organ were strongly innervated (Fig. 1l). *T. mediterranea* also showed a fine sub-epithelial nerve plexus in 5-HT stainings, which otherwise had a similar topology of the nervous system as the AVRLIRLamide stainings (see Fig. S2).

S-Phase cells and mitoses

In adult *T. mediterranea*, S-phase and mitotic cells were arranged in two broad longitudinal stripes, ranging from the anterior tip of the brain to the tail, where the stripes connected in a loop (Fig. 1n). Mitotic cells were much less numerous than cells in S-phase, with a ratio of roughly 1:10 (n = 3 animals). No proliferation was detected in the rostrum (the region anterior to the brain), the brain, the pharynx, the tip of the tail, and the epidermis. S-Phase labelling appeared weaker in the region posterior to than anterior to the pharynx (Fig. 1n).

S-Phase cell density was highest in the region posterior to the brain (33.8 ± 6.1 in 100 μm^2), followed by the tail region (20.2 ± 7.5 in 100 μm^2), and the fewest labelled cells were found in the region around the pharynx (16.9 ± 10.0 in 100 μm^2, n = 3 animals). These differences were found to be highly significant between brain, pharynx, and tail regions (one-way ANOVA, $\alpha = 0.01$, $p = 0.0004$, $F_{5.66} = 11.25$).

Regeneration capacity

We chose seven different amputation levels to test the regenerative capacity of *T. mediterranea* (see Fig. 1b). Animals were transversally amputated between the cerebral and tentacular eyes (amputation level 1, *AL1*), between the anterior and posterior lobes of the brain (*AL2*) or adjacent to the posterior end of the brain (*AL3*). Additionally, one lateral half of the rostrum was removed by a median longitudinal cut from the anterior tip through the brain, and a short transversal cut from the side (*AL4*). Other transversal amputations were performed anterior to (*AL5*), through the middle of (*AL6*), or posterior to (*AL7*) the pharynx. In general, both resulting pieces after the amputation were observed for their regeneration capacity; the anterior piece was designated as the 'posterior regenerate', and the posterior piece the 'anterior regenerate', in respect to the missing parts to be regenerated. In *AL4*, only the much larger piece containing the body and half of the rostrum was observed. In one experiment, only the piece between *AL3* and *AL5* was observed.

Amputation between cerebral and tentacular eyes

With amputation level 1 (*AL1*), mainly the capacity to regenerate tentacular eyes and the rostrum in the presence of the brain was tested ($n = 16$) (Fig. 2a).

The wound site in posterior regenerates was constricted 1 day post-amputation (dpa), forming a concave shape at the posterior tip (Fig. 2b). Four dpa, a small regenerative blastema appeared in the posterior region, and the posterior edge of the wound site started to become linear eight dpa, convex 14 dpa (Fig. 2c), and slightly smoother and rounder 21 and 35 dpa (Fig. 2d–e). At the latter time point, lateral, but not central longitudinal muscle fibres had elongated into the regenerated tissue, and there was a lack of circular muscle fibres in this tissue (Fig. 3b, arrowheads mark posterior-most circular muscle fibre). Neither brain nor pharynx was regenerated (Fig. 3d).

In anterior regenerates, the wound site had a concave shape 1 dpa (Fig. 2f). The regenerates curled up and did not move in the Petri dish at this stage. Two dpa, animals regained normal movement, a regenerative blastema appeared, and the wound site was reshaping into a convex form. The regenerating rostrum grew in length over the next days. Out of 16 animals, we observed only one individual that regenerated a single tentacular eye on the left side a week after the amputation. Fourteen dpa, small tentacular eyes started to appear in more animals from the first experimental batch (data not shown), but all ten animals from the second batch did not have eyes even 21 dpa (Fig. 2g–h). After 35 days, small tentacular eyes appeared in all animals. However, they were not aligned in two rows, but in a seemingly random pattern on the margins in the posterior region of the rostrum (Fig. 2i). A full set of muscle fibres and nerve cords was regenerated, and anterior regenerates were undistinguishable from controls at this time point (Fig. 3c, e).

Transversal amputation between brain lobes

With amputation level 2 (*AL2*), mainly the capacity to regenerate the missing parts of the brain was tested ($n = 17$) (Fig. 2j).

The posterior regenerates did not show a concave, but rather a wavelike shape at the wound site 1 dpa (Fig. 2k). Eight dpa, a convex shape was regained and retained until 21 dpa. The gut was visible posterior to the brain, extending more posteriorly 21 dpa than 14 dpa (Fig. 2l–m). First animals died at 14 dpa. After 21 dpa, no further regenerative processes were observed and all posterior regenerates were dead 35 dpa.

Upon amputation, part of the posterior brain lobes was exposed to the environment in anterior regenerates. One dpa, the brain was fully covered at the wound site (Fig. 2n). The main intestine branch was already elongated into the new tissue 8 dpa. Two weeks after amputation, eyes aligned in two longitudinal rows in front of the bisected brain started to become observable in the unpigmented regenerated tissue (Fig. 2o). It was not clear if tentacular or cerebral, or both, eye types were regenerated at this stage. The regenerated tissue was narrower than the rest of the body. Most of the anterior regenerates began to act like control animals at this time point. Twenty-one dpa, the constriction between the narrow blastema and the rest of the animal had mostly disappeared (Fig. 2p). Thirty-five dpa, an amputated animal was not distinguishable from a non-treated animal, except that the eyes were smaller. We found two pairs of cerebral eyes and two rows with tentacular eyes. Two round extensions anterior to both sides of the original brain formed the anterior lobes. The brain had regained its normal shape and size (99.8 ± 17.4 μm in length, 83.5 ± 9.4 μm in width, $n = 6$) (Fig. 2q). Actin filaments formed a capsule surrounding the brain like in control animals, also covering the newly formed anterior part of the brain (Fig. 3h). The AVRLIRLamidergic orthogonal nervous system was also fully restored at this stage, with the lateral main nerve cords forming a loop in the rostrum. Also, the ventral nerve cords anterior to the brain were fully regenerated at this stage (Fig. 3j).

Amputation posterior to the brain

With this amputation level (*AL3*), the capacity to regenerate the whole brain was tested, as well as the capacity of the posterior regenerate to rebuild the pharynx and the adhesive pad ($n = 37$) (Fig. 2r).

The posterior regenerates from this experiment retained all eyes and the complete encapsulated brain. One dpa, we observed that the wound site was constricted, thus leaving a concave shape (Fig. 2s). The unpigmented regeneration blastema was formed 2 dpa, and a continuous elongation of the part posterior to the brain was observed (Fig. 2t–v). An intestine appeared in the regenerated tissue at 14 dpa (Fig. 2t). The usually defined edges of the intestine turned into a discontinuous network in the centre of the regenerated tissue at 21 dpa (Fig. 2u). At 35 dpa, this network turned into a small regenerated pharynx (Fig. 2v). Phalloidin stainings showed the pharynx to already be strongly muscular, ruffled, and very close to the brain capsule (Fig. 3l). Also, the complete body wall musculature was restored 35 dpa. The lateral main nerve cords were connected in a posterior loop (Fig. 3n). A ring with AVRLIRL-amidergic cells appeared in the region posterior to the brain, at the location of the regenerated pharynx.

The anterior regenerates curled up and rested immobile in the Petri dish after being amputated. The wound site was constricted, leaving the anterior tip in a concave shape

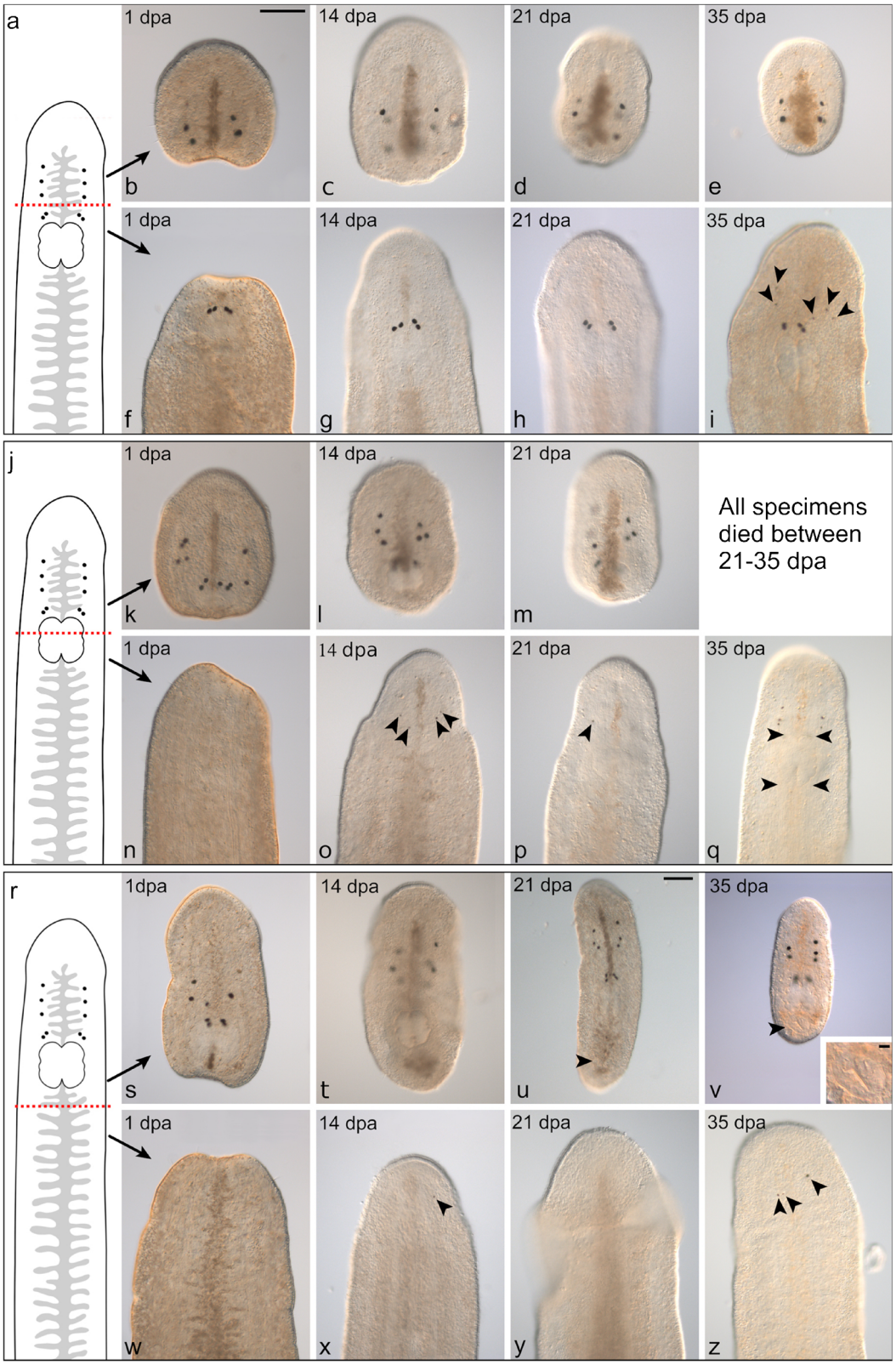

Fig. 2 Regeneration after amputation at *AL1–AL3*. **a** Schematic drawing of amputation level 1, in the head region between the tentacular and cerebral eyes. **b–e** DIC images of posterior regenerates and **f–i** anterior regenerates of animals amputated at *AL1*, respectively. **j** Schematic drawing of amputation level 2 through the brain. **k–m** Posterior regenerates and **n–q** anterior regenerates. **r** Schematic drawing of amputation level 3 posterior to the brain. **s–v** Posterior regenerates and **w–z** anterior regenerates. Inset in **v** shows the new pharynx in a higher magnification. *Scale bar* in **b** (100 μm) applies to all panels except **u** (also 100 μm); inset: 10 μm

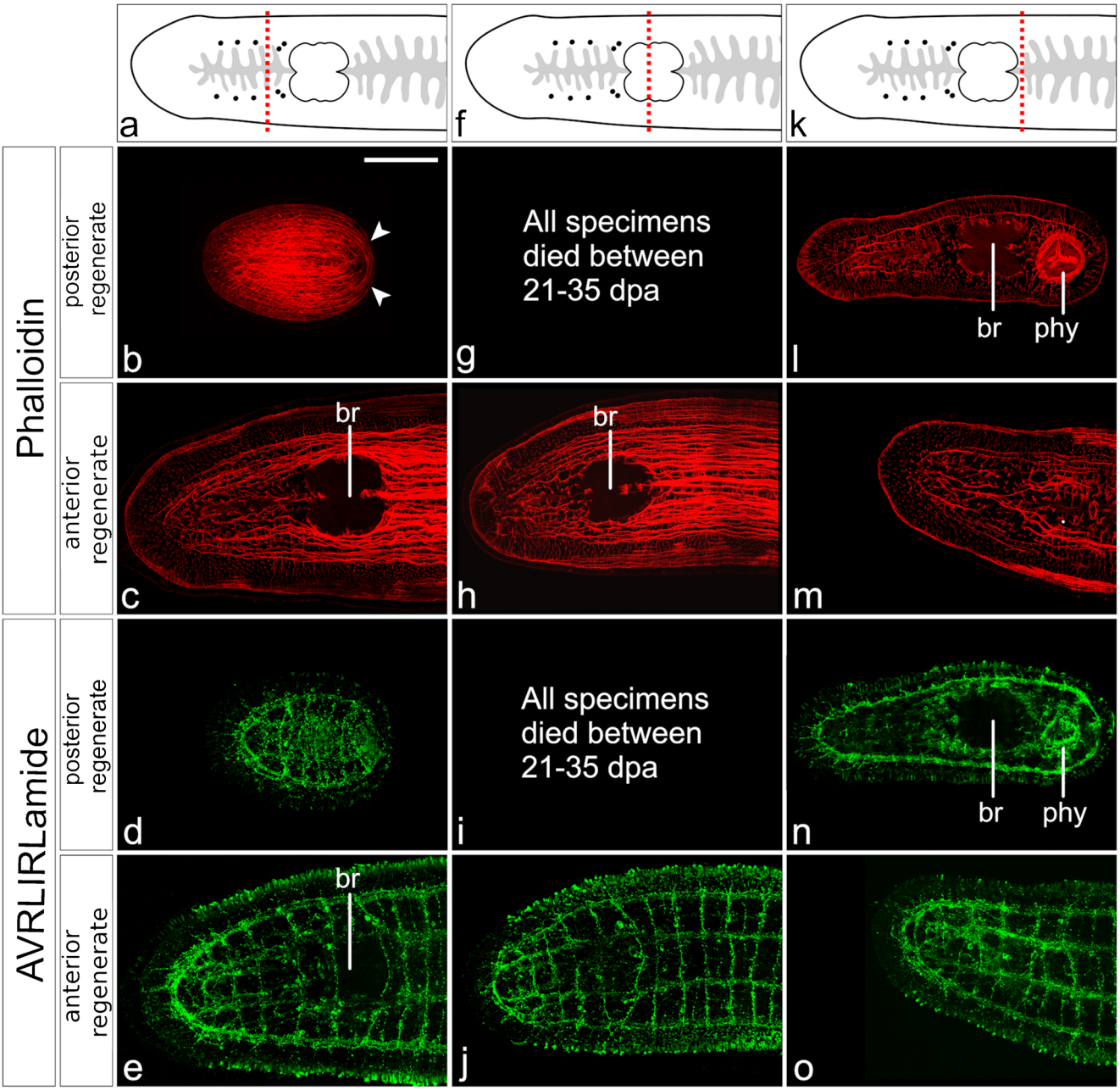

Fig. 3 F-Actin filaments and AVRLIRLamidergic nervous system after regenerating for 35 days. **a**, **f** and **k** Schematic drawings of the amputation level. **b**–**e** Regeneration of the musculature and nervous system after a transversal amputation at *AL1* in posterior (**b**, **d**) and anterior (**c**, **e**) regenerates, respectively. **h**–**j** As precedent, but with *AL2*. **l**–**o** As precedent, but with *AL3*. *Scale bar*: 100 μm

1 dpa (Fig. 2w). The wound site bulged out and started to grow in length 14 dpa (Fig. 2x), and in one specimen, a single eye in the regenerated tissue could be observed. At this point, the animals moved normally through the Petri dish, performed the typical movement with their head and the reaction to other animals as well as to light was not distinguishable from non-amputated control animals. One week later, no additional animals with regenerated eyes were detected (Fig. 2y). Two out of seven, and only one out of ten, animals (from the first and third batch, respectively) were able to regenerate several eyes at 35 dpa (Fig. 2z). The brain was not regenerated to any extent, and also in phalloidin stainings there was no indication of a brain capsule (Fig. 3m). We found a typical pattern of all other muscles in the rostrum. Despite the missing brain, lateral nerve cords formed a rostral loop (Fig. 3o). Also, an orthogon with irregular dense fibres and cell bodies was visible in the regenerated tissue.

Lateral amputation through the brain

With this amputation level (*AL4*), we tested if the lateral half of the brain is sufficient to regenerate the missing parts ($n = 10$) (Fig. 4a).

By taking pictures directly after amputation, we ensured that all cut animals had only half the brain remaining (Fig. 4b). We observed that all animals were able to regenerate two cerebral eyes as well as two to five tentacular eyes within 58 days of regeneration (Fig. 4c). Observed animals looked and acted like control animals. The regenerated half of the rostrum was only distinguishable from the remaining half, because the regenerated eyes were much smaller than the remaining eyes. The missing brain half was not regenerated at 58 dpa.

Amputation anterior to the pharynx

Amputation level 5 (*AL5*) tested if the pharynx would be regenerated after complete removal of the pharynx (Fig. 5a).

In posterior regenerates, a new pharynx was regenerated *de novo* in a region anterior to the blastema, i.e. in the old tissue remaining after amputation (Fig. 5a). Two dpa, animals did not show any sign of a regenerating pharynx in live images (Fig. 5b) or stainings of the musculature (Fig. 5c) and the nervous system (Fig. 5d). The orange-brown intestine was interrupted by a collar indicating the pharyngeal primordium 7 dpa (Fig. 5e). Longitudinal muscle filaments were slightly bent around the pharyngeal primordium, and the circular muscles around the intestine were gone in this region (Fig. 5f). Small actin filaments started to grow radially from its margins inwards. The transversal commissures of the orthogon were interrupted in the area of the pharyngeal primordium, and the main nerve cord pair was bent around it (Fig. 5g). Nine dpa, the darkish colouration of the intestine was divided into two parts, with the pharyngeal primordium embedded in between (Fig. 5h). Visualisation of F-actin showed the pharynx primordium taking the shape of a pointed donut (Fig. 5i), where the outer margin as well as the inner margin of the primordium was surrounded by muscles. Haphazardly arranged thin actin filaments formed a fine mesh in between both margins. The pharyngeal primordium was almost devoid of serotonin-positive signal (Fig. 5j). After almost 2 weeks, the regenerating pharynx showed a longitudinal slit as its lumen (Fig. 5k). The actin filaments appeared more organised, and the outermost margin of the regenerating pharynx was enclosed by an ample layer of circular muscles. Thin actin filaments ran radially between the outer ring and the inner slit of the regenerating pharynx (Fig. 5l). The serotonergic nervous system grew thin fibres into the pharynx (Fig. 5m).

The anterior regenerates were not able to regenerate any organs after the amputation. Apart from a connection of the longitudinal muscles and the ventral main nerve cords through a thin serotonin-positive commissure in front of the original pharynx at 4 dpa, no visible structures were regenerated. Three-week-old anterior regenerates looked similar to two dpa stages (Fig. S3).

Double amputation between posterior of the brain and anterior of the pharynx (*AL3 and AL5*)

With simultaneous amputation levels *AL3* and *AL5*, the capacity to *de novo* regenerate the brain, the pharynx, and the adhesive pad was tested ($n = 10$) (Fig. 6a).

We could distinguish three different outcomes after a regeneration time of more than 45 days. In the majority of observed animals (six out of ten), the specimens were able to regenerate a pharynx and an adhesive pad at the posterior

Fig. 4 Regeneration capacities if amputated at amputation level 4 (*AL4*). **a** Schematic drawing of *AL4*; only the large piece of the amputated animal was observed. **b** The longitudinally incised animal at 0 dpa, before one-half of the rostrum was cut away as indicated in **a**. **c** The regenerated lateral rostrum, including two cerebral and one tentacular eye (in another focal plane, arrowheads), at 58 dpa. However, the missing half of the brain was not regenerated (double arrowheads). *Scale bars* 100 μm

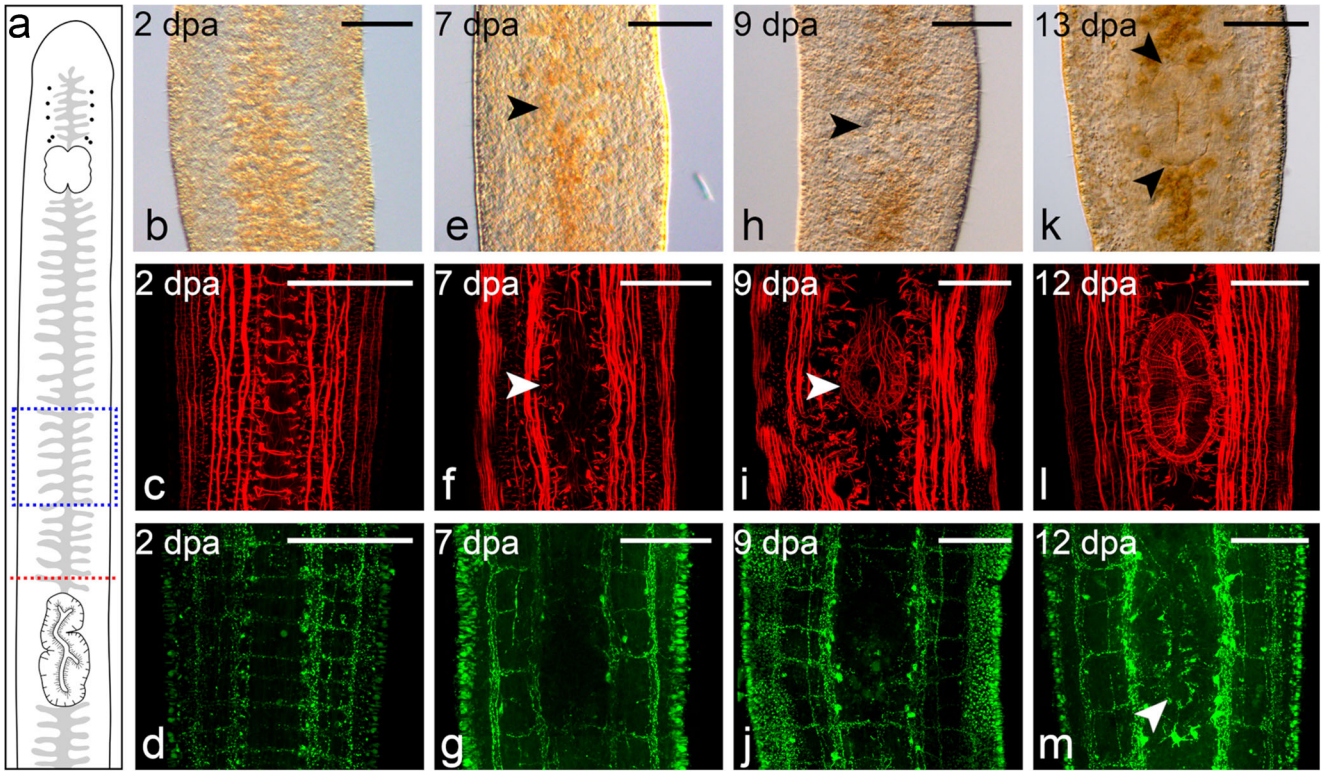

Fig. 5 Live observations, phalloidin and 5-HT as markers for ongoing pharynx regeneration. **a** Schematic drawing of *AL5*; blue box roughly shows the position where the new pharynx will appear. **b–d** Live observation (**b**), F-actin (**c**) and 5-HT (**d**) stainings showed an unaltered arrangement of muscle and nerve fibres at 2 dpa. **e–g** Irregularity in the intestine hints at the position of the pharyngeal primordium at 7 dpa (**e**). Circular muscles around the intestine disrupted and longitudinal muscles slightly bend around pharyngeal primordium (**f**). At 9 dpa, pharyngeal primordium is well visible (**h–j**). Differentiated pharynx after 12–13 dpa (**k–m**). *Scale bars* 100 μm

end (Fig. 6b). A second result was a regenerated adhesive pad at the posterior tip, but lacking a pharynx (two out of ten, Fig. 6c). In a third outcome, two out of ten animals had regenerated adhesive pads on both ends as well as two pharynges in the respective halves of the specimens (Fig. 6d–f).

Amputation through the pharynx

Amputation level 6 tested if the missing part of the pharynx would be regenerated, or if the whole pharynx would be regenerated *de novo* ($n = 10$) (Fig. 6g).

After cutting, the pharynx immediately stuck out from the wound site in posterior regenerates. One dpa, the pharynx was retracted into the inside the pharynx sheath (Fig. 6h). The translucent regenerative blastema became visible 2 dpa, and the newly formed tissue grew further in length 4 dpa (Fig. 6i). The missing part of the pharynx regenerated from the remaining part of the pharynx. A newly regenerated intestine became visible at 6 to 8 dpa (Fig. 6j). After nearly 2 weeks, the pharynx and the tail, including the adhesive organ, were fully regenerated (Fig. 6k).

The halved pharynx also stuck out of the wound site in anterior regenerates directly after cutting, but after 24 h, the pharynx was retracted again into the remaining pharynx sheath (Fig. 6l). We could observe a small regenerative blastema 3 dpa. The anterior-most tip of the wound site regained a smooth round shape 4 dpa (Fig. 6m). We could not detect any changes after this stage (Fig. 6n–o).

Stem cell dynamics during regeneration

The following experiments monitored the proliferation activity in regenerating animals, with emphasis on the posterior blastema and the pharynx primordium.

Stem cell dynamics in the regenerating pharynx and genital primordium

In these experiments, we repeated cutting at *AL5* to follow proliferating cells during pharynx regeneration in the posterior regenerate (Fig. 7a). S-Phase neoblasts were labelled with the thymidine analogue EdU (pulse-chase), kept for regeneration for either 7 or 14 days, and labelled a second time with the thymidine analogue BrdU (pulse) before fixation. The negative controls, where the BrdU or the EdU label was omitted, revealed staining of rhabdites and other gland cells within the epidermis of the animal (Fig. S4).

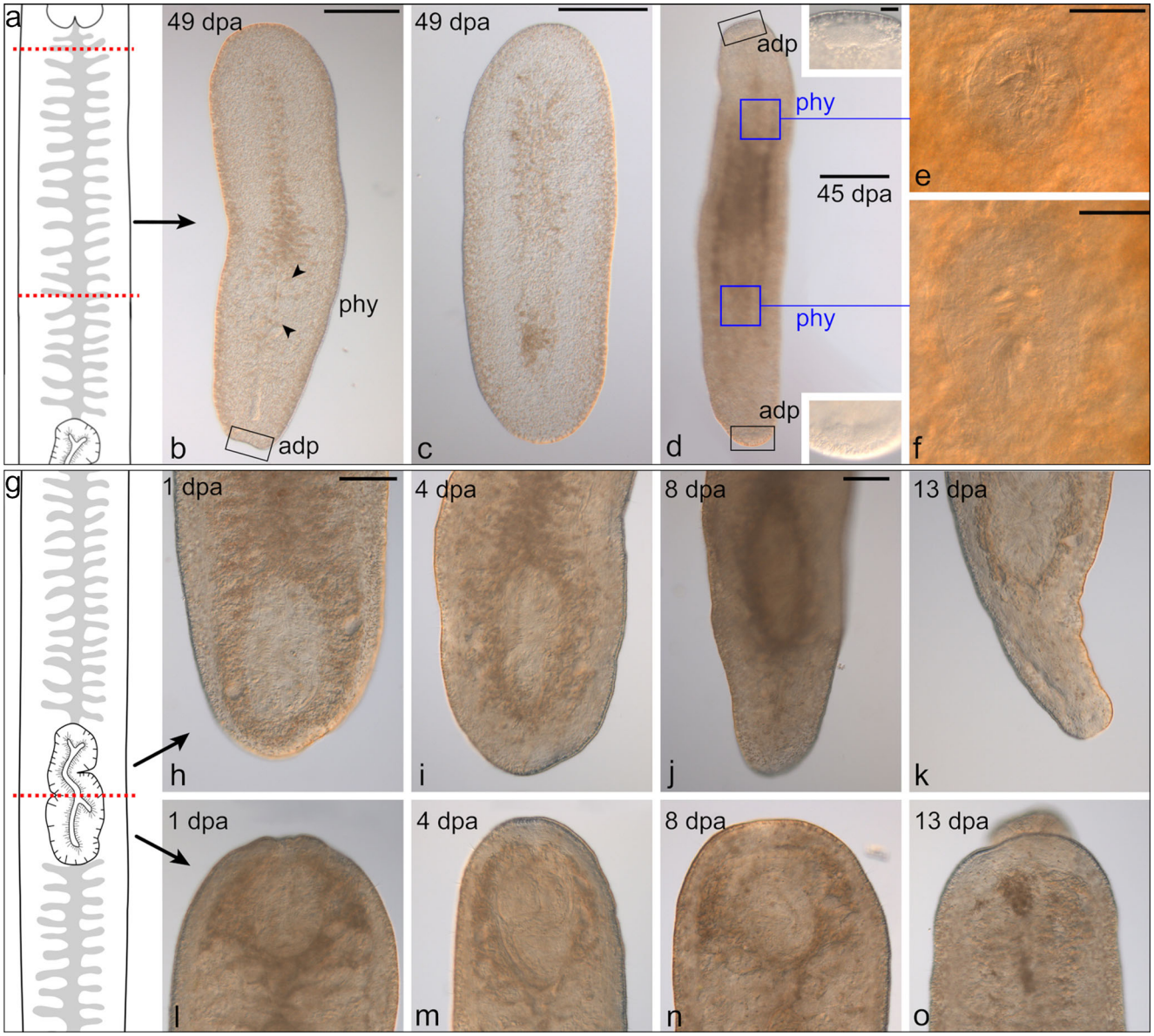

Fig. 6 Regeneration after amputation at *AL3* and *AL5* and *AL6*. **a** Schematic drawing of the piece resulting from amputation between *AL3* and *AL5*, with one of three outcomes (**b–f**). The piece shown in **b** shows a regenerated pharynx *phy* (arrowheads) and an adhesive pad *adp* (inset) ($n = 6/10$), the piece in **c** has one *adp*, but no *phy* ($n = 2/10$), and the piece in **d** includes two *adp* (insets show detail of framed pieces) and two *phy* ($n = 2/10$). Details of both pharynges from **d** shown in **e** and **f**. **g** Schematic drawing of the piece resulting from amputation at *AL6*. DIC images of the posterior regenerates (**h–k**) and of anterior regenerates (**l–o**), respectively. *Scale bars* b–d: 200 μm; h, i, k–o: 100 μm; j: 100 μm; e–f: 50 μm; insets in d: 20 μm

Seven dpa, the broad longitudinal stripes of S-phase neoblasts were spread around the pharyngeal primordium (Fig. 7c). The centre of the primordium was free of S-phase cells. However, the left and right margins of the primordium showed an aggregation of S-phase neoblasts. Chased stem cells were found in the posterior tip of the animal and revealed some of the newly regenerated tissue after the amputation (Fig. 7b). The chased cells were still arranged in two longitudinal stripes, which appeared broader than in the pulsed cells. A cluster of chased cells appeared in the pharyngeal primordium (Fig. 7b). An overlay of pulsed and chased cells highlighted their different distribution, surrounding and within the pharynx primordium, respectively (Fig. 7d).

A similar pattern was visible in 14-dpa specimens. The diameter of the ring around the pharynx was increased, and more S-phase neoblasts clustered around it (Fig. 7f). At this stage, the longitudinal stripes were not visible anymore in the chased cell pattern (Fig. 7e). The signal appeared evenly distributed throughout the whole animal except for the

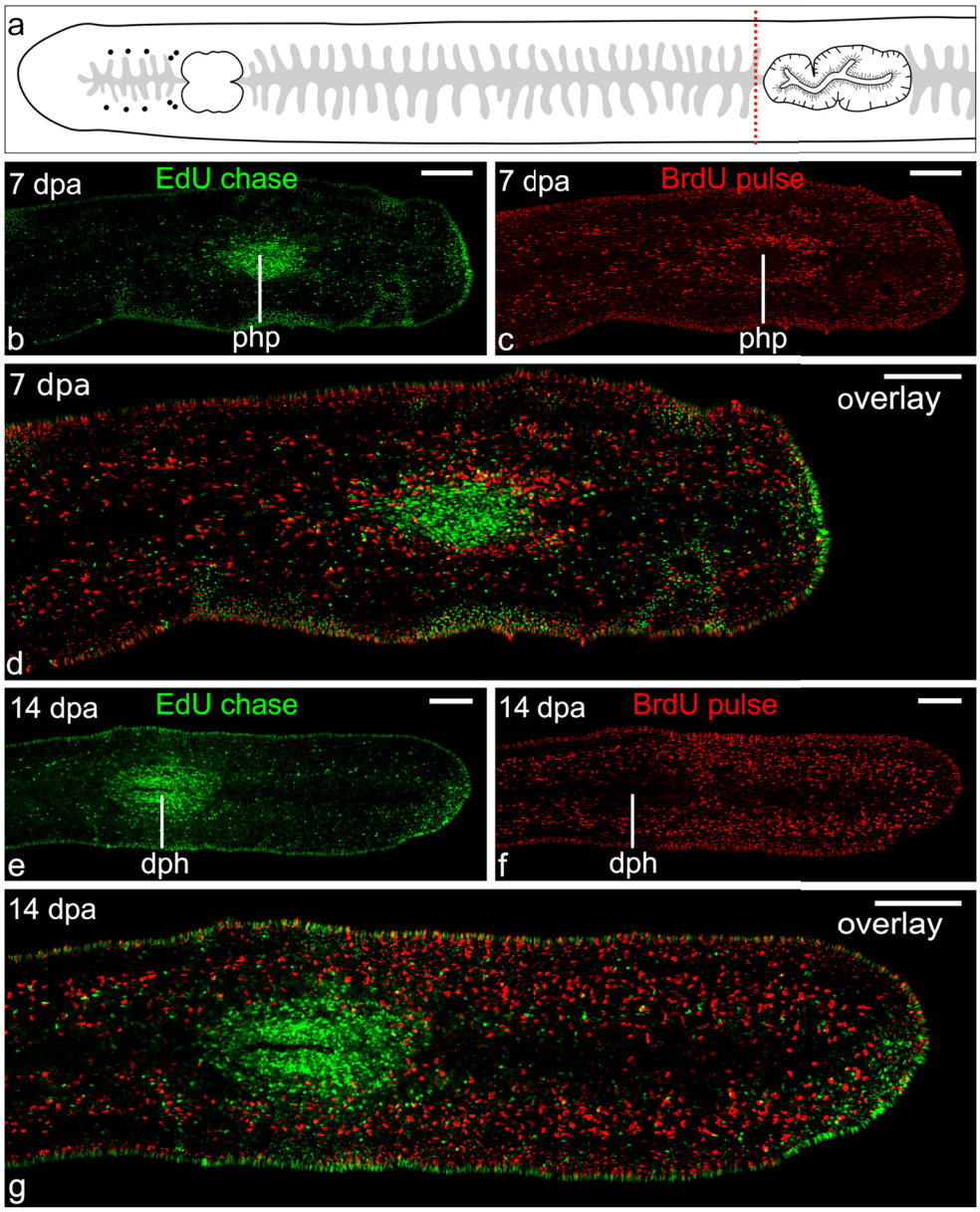

Fig. 7 Stem cell dynamics after amputation at *AL5*. **a** Schematic drawing of *AL5*. EdU chased (**b**) and BrdU pulsed (**c**) 7 dpa posterior regenerates. **d** Overlay shows pharyngeal primordium (*php*) with a patch of chased nuclei (green) in the middle, surrounded by pulsed nuclei (red). **e**–**g** As above, but after 14 days, with a differentiating pharynx (*dph*). *Scale bars* 100 μm

accumulation in the regenerated pharynx and the posterior tip. Also, the chased cells filled the complete regenerated pharynx, including its sheath. The overlay revealed that no S-phase neoblasts were found within the regenerated pharynx (Fig. 7g).

Both at 7 and 14 dpa, anterior regenerates showed a cluster of chased cells in the anterior tip of the animals (Figs. S5b, e). The S-phase neoblasts were aligned in two stripes. The regenerative blastema in the anterior end of the amputated specimens showed an aggregation of S-phase neoblasts. Chased cells ranged into the very tip of the regenerative blastema, whereas the S-phase neoblasts did not reach thus far (Figs. S5c, f)

A second big cluster with an ovoid shape was found at the region of the putative genital complex, which was surrounded by S-phase neoblasts, leaving the centre of the primordium empty of S-phase neoblasts but full of chased cells (Fig. S5d, g). The stem cell accumulation in the region of the male copulatory organ is not due to the regeneration process induced by amputation, as the male copulatory organ was never amputated in these regenerates.

Stem cell dynamics in the regeneration blastema

After amputation posterior to the pharynx (*AL7*) and a subsequent regeneration time of 3 to 8 days, we labelled

S-phase neoblasts with the thymidine analogue EdU prior to fixing the specimens. The visualised S-phase neoblasts revealed proliferation within the regenerative blastema at four time points in anterior regenerates (data not shown) and in posterior regenerates (Fig. 8a).

The posterior regenerates showed an accumulation of S-phase neoblasts in a 3-day-old regenerative tissue (Fig. 8b). The overall pattern of stem cells showed no difference between regenerates and controls ($n = 6$). Two broad longitudinal stripes of S-phase neoblasts ranging from the anterior margin of the brain to the tip of the blastema were visible. S-Phase neoblasts were clustered around the median region posterior to the pharynx, which was free of stem cells in control animals. Four dpa, S-phase neoblasts were accumulating in the whole regeneration blastema (Fig. 8c). Twenty-four hours later, proliferation in the posterior part of the regenerate, including the blastema, continued to be high (Fig. 8d). The very tip of the newly regenerated posterior end was free of stem cells. Starting at 5 dpa and clearly visible at 8 dpa, the S-phase neoblast cluster was split in half and an intestine was most probably formed in between (Fig. 8e). An accumulation of S-phase neoblasts in the regenerated tissue was still taking place, while the posterior-most tip lacked S-phase neoblasts at this time point.

Discussion

Polyclad regeneration research is pervaded by studies with a low level of detail and with unclear or contradictory results even for closely related species (see Tables 1 and 2). Here, we provide a synopsis of past research, integrating our own data with previous accounts to formulate a guide of what and how polyclads can (or cannot) regenerate.

Taxonomic and systematic considerations

Charles Manning Child performed his regeneration experiments with an undescribed animal he found in ‘considerable numbers in fine sand’ in Naples and which he tentatively determined as ‘*Cestoplana* sp.’ in 1905 (Child 1905b). The genus *Theama* was only erected in 1949 (Marcus 1949), but Child’s description and drawings strongly suggest that he actually worked with *Theama mediterranea*, a species occurring throughout the Mediterranean (Curini-Galletti

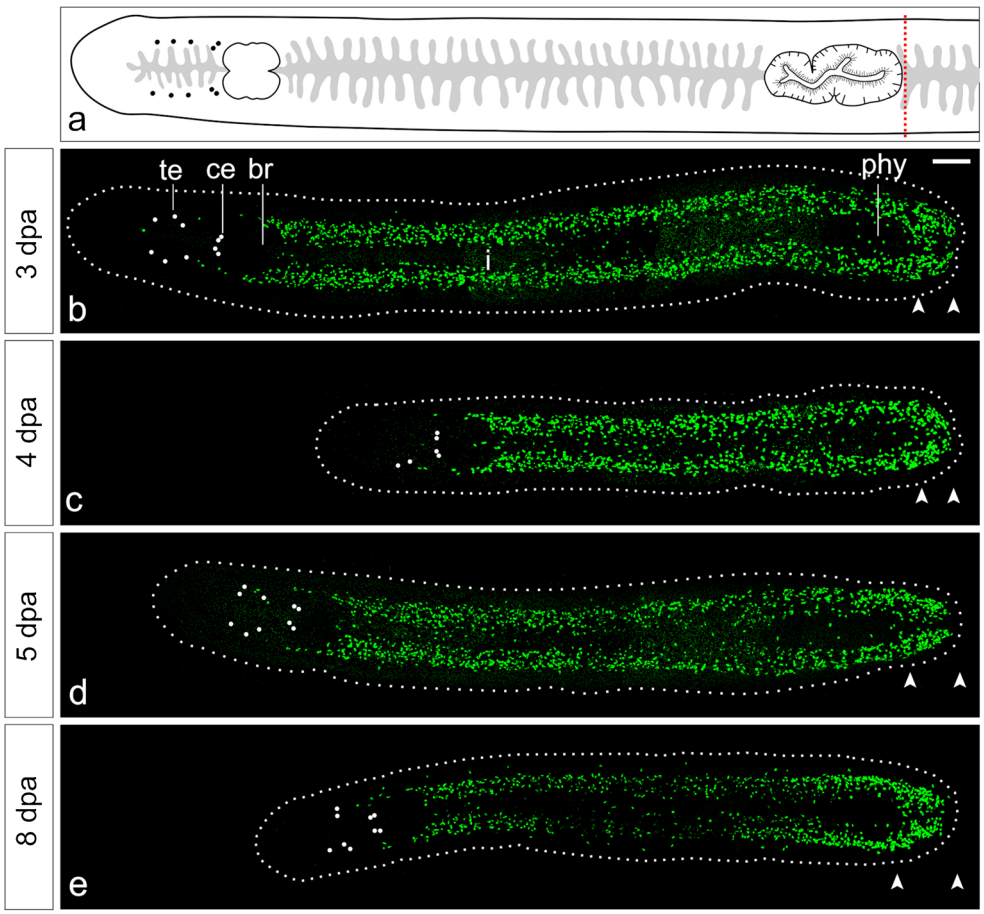

Fig. 8 Stem cell dynamics after amputation at *AL7*. **a** Schematic drawing of *AL7*. EdU pulsed S-phase neoblast stem cells after 3 (**b**), 4 (**c**), 5 (**d**) and 8 days (**e**), respectively. Arrowheads indicate the extension of the posterior blastema. *br*, brain; *ce*, cerebral eyes; *phy*, pharynx; *te*, tentacular eyes. All eyes are indicated with white dots in **b**–**e**, and outlines of the specimens delineated by dotted lines. *Scale bar* 100 μm

Table 1 Regeneration capabilities in acotylean polyclads. Groups *(Grp)* as outlined in the Discussion

Acotylea				
Family/species	Grp	Regeneration capacity	Data availability	Authors
Cryptocelidae				
Cryptocelis alba	II	'Like Thysanozoon'	Medium; some drawings, otherwise 'like Thysanozoon'	Monti (1900)
Hylocelis californica	III	Cannot completely regenerate when cut just posterior to brain; Cannot regenerate lateral half of brain; Can regenerate anterior to brain; Cannot regenerate brain; Can regenerate posterior tip in absence of brain	High; several cutting levels	Olmsted (1922)
Discocelis tigrina	I	Slow and incomplete	Poor; just mentioned in the text	Monti (1900)
Leptoplanidae				
Leptoplana tremellaris	IV	Lang: 'can regenerate pharynx posteriorly'; Monti: 'like Thysanozoon' Child: anterior and posterior to brain can regenerate; parts of ganglia can regenerate anteriorly; complete brain does not regenerate; lateral half of brain can regenerate von Levetzow: 'like Thysanozoon'	Lang: medium; one drawing, short description Monti: poor; no drawings, otherwise 'like Thysanozoon' Child: high; many cutting levels, descriptions, many drawings von Levetzow: medium; some descriptions and figures, but not comprehensive	Lang (1884); Monti (1900); Child (1904a, b, c, 1905a); von Levetzow (1939)
Leptoplana littoralis	III	Everything but the brain	High; several cutting levels, drawings; histology	Morgan (1905)
Undetermined leptoplanid	III	Not only the brain capsule, but also the nerve roots are important for anterior (eye) regeneration; no regeneration of the whole brain	High; several cutting levels, drawings, descriptions	Child (1910)
Undetermined *Leptoplana*	II	Cannot regenerate anteriorly, i.e. also cannot regenerate head; posterior parts regenerate, like genital organs and sucker	Medium; just two cutting levels in the middle of the body (anterior + posterior of pharynx), histology, several figures, detailed descriptions	Schultz (1901, 1902)
Notoplanidae				
Notoplana alcinoi	III	Monti: 'like Thysanozoon' von Levetzow: 'like Thysanozoon'	Monti: medium; one drawing, otherwise 'like Thysanozoon' von Levetzow: medium; some descriptions, but not comprehensive	Monti (1900) von Levetzow (1939)
Notocomplana litoricola	III	Cannot completely regenerate when cut just posterior to brain Cannot regenerate lateral half of brain Can regenerate anterior to brain Cannot regenerate brain Can regenerate posterior tip in absence of brain	High; several cutting levels, drawings	Olmsted (1922)

Table 1 (continued)

Acotylea				
Family/species	Grp	Regeneration capacity	Data availability	Authors
Notocomplana saxicola	III	Cannot completely regenerate when cut just posterior to brain Cannot regenerate lateral half of brain Can regenerate anterior to brain Cannot regenerate brain Can regenerate posterior tip in absence of brain	High; several cutting levels	Olmsted (1922)
Notocomplana humilis	IV	Can regenerate anterior, but not posterior half of brain Cannot regenerate whole brain	Ishida: high; several cutting levels, drawings Okano et al.: high; drawings, photographs, stainings	Ishida (1998) Okano et al. (2015)
Stylochidae				
Stylochus pilidium	I	Slow and incomplete	Poor; just mentioned in the text	Monti (1900)
Stylochus neapolitanus	I	Only peripheral body parts	High; with drawings and text	von Levetzow (1939)
Stylochoplanidae				
Comoplana agilis	III	'Like Thysanozoon'	Medium; some descriptions and figures, but not comprehensive	von Levetzow (1939)

et al. 2008; Gammoudi et al. 2017). While both genera share an elongated body shape, the following considerations support our conclusion that Child's '*Cestoplana*' is in fact *Theama mediterranea* (for comparison, see Fig. S6). Most obviously, Child has drawn the pharynx in the middle of the body (Child 1905b, c), which is highly unusual for *Cestoplana*, where the pharynx is situated in the posterior third (Lang 1884; Marcus 1949; Sopott-Ehlers and Schmidt 1975; Oya and Kajihara 2019). Second, Child describes his experimental species as 'semitransparent' and unpigmented; *T. mediterranea* is greyish-transparent (Curini-Galletti et al. 2008; Gammoudi et al. 2017), while *Cestoplana* usually shows a distinct colour pattern (Lang 1884; Oya and Kajihara 2019). With the exception of the eyeless bathyal species *Cestoplana nopperabo* (Oya and Kajihara 2019), *Cestoplana* usually has hundreds of eyes in a large continuous patch in the head (Lang 1884; Marcus 1949; Sopott-Ehlers and Schmidt 1975). Child reports only six eyes (with frequent variations of numbers) in two rows in his species (Child 1905b), in a very similar pattern as in *T. mediterranea* (Curini-Galletti et al. 2008) with usually ten eyes, but see Fig. S1 for variations. Finally, so far no *Cestoplana* species has been described that matches Child's description of '*Cestoplana*', which like *Theama mediterranea* was found in high numbers (Curini-Galletti et al. 2008; Gammoudi et al. 2017), whereas *Cestoplana* species are only found in low abundance (Lang 1884; Marcus 1949; Sopott-Ehlers and Schmidt 1975; Oya and Kajihara 2019).

So far, 23 species of polyclads have been scrutinised regarding their regeneration capacity in 19 studies (Tables 1 and 2). Of these, 14 species from five families belong to Acotylea, and nine species from four families to Cotylea. The acotylean *Leptoplana tremellaris* is the best-studied species with seven dedicated works (Lang1884; Monti 1900; Child 1904a, b, c; 1905a; von Levetzow 1939), but recently it was found that *L. tremellaris* is a species complex comprising at least two species, *L. tremellaris* and *L. mediterranea* (Gammoudi et al. 2012). *Leptoplana atomata* used by Schultz (1901, 1902) was revised as *Pleioplana atomata* (Faubel 1983), but the genital pit present in Schultz' descriptions is characteristic of the genus *Leptoplana* and missing in *Pleioplana*—therefore, it is not clear what species of *Leptoplana* Schultz was working with. While the acotylean families Leptoplanidae and Notoplanidae have the largest number of studied representatives, they are notoriously hard to determine: two of four species of studied Leptoplanidae remain undetermined, and all of the listed Notoplanidae were taxonomically revised and renamed (see Table 1).

As well, *Cestoplana* and *Theama* were classified as acotyleans (Faubel 1984; Prudhoe 1985), but recent molecular phylogenies recover both as cotyleans (Bahia et al. 2017; Oya and Kajihara 2019). Thus, the best-studied cotyleans are

Table 2 Regeneration capabilities in cotylean polyclads. Groups *(Grp)* as outlined in the Discussion

Cotylea Family/species	Grp	Regeneration capacity	Data availability	Authors
Eurylepidae				
Stylostomum ellipse	I	Slow and incomplete	Poor; just mentioned in the text	Monti (1900)
Eurylepta cornuta	III	Can regenerate head with small tentacles, but no details shown	Poor; rudimentary description, no figures shown	Dalyell (1853)
Maritigrella crozieri	I	Only posterior blastema and regenerating periphery shown	Poor; one cut in middle of body, some photographs	Lapraz et al. (2013)
Prosthiostomidae				
Prosthiostomum dohrni	II	Regenerates posterior to pharynx	Poor; just observation, drawing	Lang (1884)
Prosthiostomum siphunculus	I	Slow and incomplete	Poor; just mentioned in the text	Monti (1900)
Pseudocerotidae				
Thysanozoon brocchi	III	Monti: can regenerate lateral half, including complete pharynx and smaller tentacle; after transversal cut, does not regenerate head; regenerates genitals and sucker in absence of head von Levetzow: cannot regenerate brain after only removing the brain; tentacles regenerate if brain present; pharynx, main gut, sucker, and genitals can regenerate after complete removal, even in absence of brain; if only pharynx is removed, pharynx does not regenerate properly	Monti: high; several cutting levels, drawings many experiments, many pictures von Levetzow: high; many cutting levels, histology, drawings, text	Monti (1900), von Levetzow (1939)
Pseudoceros velutinus	I	Slow and incomplete	Poor; just mentioned in the text	Monti (1900)
Yungia aurantiaca	I	Slow and incomplete	Poor; just mentioned in the text	Monti (1900)
Theamatidae				
Labelled as '*Cestoplana* sp.', but most likely *Theama mediterranea*	IV	Child: can regenerate even the whole brain if amputated just posterior to brain present study: can only regenerate anterior half of brain	High; many cutting levels, drawings, descriptions	Child (1905b, c, d), present study

Theama mediterranea (this work, Child 1905b, c, d) and *Thysanozoon brocchi* (Monti 1900; von Levetzow 1939). These are also the only two cotyleans that have received a comparatively high level of experimental detail (see Table 2).

Summary of *Theama*'s regeneration capacity

In the present study, *Theama mediterranea* was found to be able to regenerate the rostrum including eyes anterior to the brain, and even the anterior (but not the posterior or lateral) half of the brain. In an amputation level just posterior to the brain, the posterior regenerate could regenerate a pharynx within 35 days, whereas the anterior regenerate could not regenerate the brain or the complete rostrum, but sometimes a few eyes. The pharynx cut in half did fully regenerate in the posterior, but not in the anterior regenerate. A middle piece cut behind the brain and anterior to the pharynx could regenerate a pharynx and adhesive pad in the absence of the brain—sometimes, even two pharynges and two adhesive pads on opposing sides of the middle piece. For

a schematic overview of *T. mediterranea*'s regeneration capacity, see Fig. 9.

Not all polyclads follow the polyclad rule of regeneration

Most polyclads that were thoroughly experimentally tested for their regeneration capacity were reported to regenerate all body parts except the (complete) brain (Tables 1 and 2). This includes major organs like pharynx, genital complex, and the sucker (see e.g. Monti, 1900; von Levetzow, 1939). The rule for polyclad regeneration states that polyclads are 'able to restore missing parts, provided the cephalic ganglia are intact'. If the whole brain is removed, then 'regeneration cannot take place anteriorly, though it may do so posteriorly' (Olmsted 1922).

Three acotyleans and five cotyleans are in conflict with this polyclad rule for regeneration in that they cannot regenerate any organs (see Tables 1 and 2). Only one of the concerned studies provides some detail in text and figures: *Stylochus neapolitanus* can only regenerate small peripheral body parts (von Levetzow 1939), which is corroborated by an earlier and less detailed study that found that *Stylochus pilidium* is not able to regenerate to any extent. Stylochidae is thus the only studied family in which all observed representatives are poorly regenerating. *Discocelis tigrina* is the odd worm out in the family Cryptocelidae, in which the two other studied representatives can regenerate well. However, most polyclads that were found to regenerate poorly were dealt with in a single paper, and the author mentioned that possibly the culture conditions were responsible for low regeneration capacity in some polyclads (Monti 1900). This may be the case in the genus *Prosthiostomum*, where *P. dohrni* was noted to have regenerated the posterior half (Lang 1884), whereas its congener *P. siphunculus* was not found to regenerate much (Monti 1900). The euryleptid *Maritigrella crozieri* was only superficially observed and was found to regenerate all peripheral body parts, but after 16 days, only a relatively small piece of regenerated tissue in a posterior regenerate had formed (Lapraz et al. 2013).

The whole brain cannot regenerate in polyclads

In some cases, a regeneration capacity exceeding the rule for polyclad regeneration was reported. Already the first study about polyclad regeneration states that *Eurylepta cornuta* can regenerate 'head and horns', i.e. much of the anterior part of the animal including tentacles (Dalyell 1853); however, no details or figures are given, and neither brain nor eyes are mentioned. In two cases, histological sections have been provided to document the lack of regeneration of the brain: in *Leptoplana littoralis* (Morgan 1905) and in

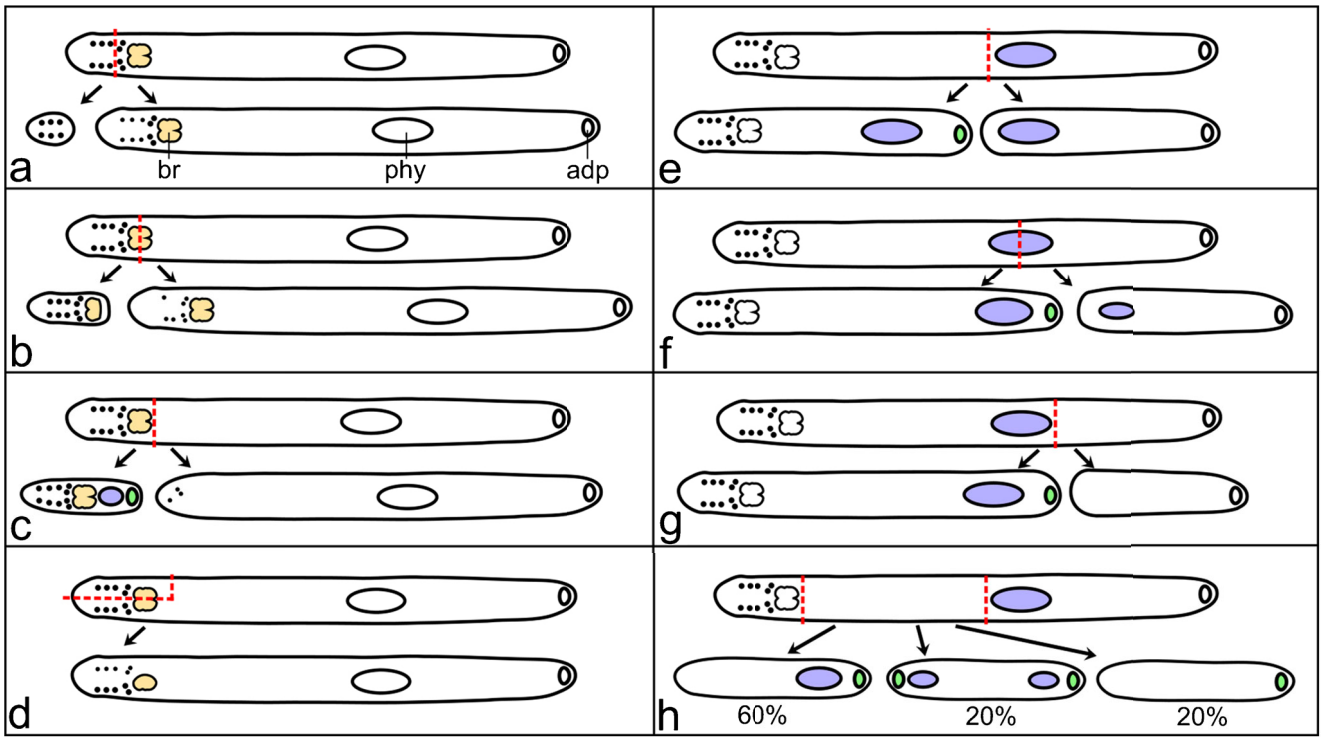

Fig. 9 Regeneration capacity of *T. mediterranea.* Synopsis of the regeneration capacity of *T. mediterranea* after transversal amputation at amputation level 1 (*AL1*): (**a**), *AL2* (**b**), *AL3* (**c**), *AL4* (**d**), *AL5* (**e**), and *AL6* (**f**), *AL7* (**g**), double amputation at *AL3* and *AL5* (**h**). *adp*, adhesive pad: *br*, brain; *phy*, pharynx

Thysanozoon brocchi (von Levetzow 1939), it was shown that the main nerve fibres reconnect, but a brain capsule is not formed anymore. Our nervous system stainings with *Theama* confirm these results (Fig. 3o).

Only one species, the aforementioned '*Cestoplana* sp.' was comparatively thoroughly documented to regenerate even the brain, if cut just posterior to the brain (Child 1905b). Here, we made the case that '*Cestoplana* sp.' is most likely *Theama mediterranea*, and that this species does not regenerate the whole brain in our experiments (Figs. 2w–z, and 3m, o). In the original treatise by Child, only drawings of live observations were provided, and in two of eight specimens cut behind the brain, a tiny regenerated brain was noted 12 and 25 days after amputation (Child 1905b). The supposed brain was drawn always immediately adjacent to the anterior tip of the main intestine branch and it is conceivable that bubbles in the intestine gave the impression of a small brain as drawn by Child. We scrutinised 37 specimens cut just posterior to the brain using both live observations and fluorescence stainings and found no signs of brain regeneration in any of them (see Figs. 2 and 3).

Only the anterior part of the brain can regenerate in some polyclads

If the brain was cut horizontally (see Fig. 2j), the anterior part of the brain was found to regenerate in two acotylean species, namely *Leptoplana tremellaris* (Child 1904b, c) and *Notocomplana humilis* (Ishida 1998). Also, in the present study, *Theama mediterranea* was found to regenerate parts of the brain anteriorly using live observations (Fig. 2q) and confirmed with stainings (Fig. 3h, j). Posteriorly, the cut brain did not regenerate the missing parts of the brain in *T. mediterranea* (Fig. 2k–m) or any other studied polyclad, including *L. tremellaris* (Child 1904b) and *N. humilis* (Ishida 1998). Apparently, there are cells in the posterior part of the brain that cannot be restored, suggesting that these cells may be unique in the polyclad body or important for directing regenerative response (Child 1910).

Mixed results have been published concerning the regeneration of the lateral half of the brain, which take place neither in *T. mediterranea* (Fig. 4), nor in *Hylocelis californica*, nor in *Notocomplana litoricola*, nor in *Notocomplana saxicola* (Olmsted 1922), but was reported in *Leptoplana tremellaris* (Child 1904a, 1905a). Child presented drawings and descriptions of the regenerated lateral brain half, but did not provide histological sections or stainings. In *Thysanozoon brocchi*, a longitudinally halved animal supposedly completely regenerated both lateral pieces, but no further detail was given (Monti 1900). In both cases, it is rather doubtful if the lateral half of the brain actually regenerated.

Most organs can regenerate without the brain

In the absence of the brain, *Theama* was able to regenerate or build eyes (Fig. 2z), muscle fibres (Fig. 3m), and nerve fibres (Fig. 3o) in anterior regenerates, and a pharynx (Fig. 6 b, see also Child, 1905d), an adhesive pad (Fig. 6b), and a putative genital primordium (Fig. S5) in posterior regenerates.

Similar results have been obtained in *Thysanozoon brocchi* (Monti 1900; von Levetzow 1939) and in *Leptoplana tremellaris* (Child 1904b), where the pharynx, the complete genitals, and, in the case of *T. brocchi*, also the sucker were regenerated in pieces lacking the brain. In contrast, two other studies on leptoplanids concluded that no regeneration to the anterior took place in the absence of the brain (Schultz 1901; Morgan 1905). In a follow-up paper, Child made a case that anterior regeneration in the absence of the brain or any remaining tissue anterior to the brain is indeed possible in *Leptoplana* (Child 1910).

In *T. brocchi*, only tentacles and eyes could not regenerate completely without brain: regenerated tentacles were smaller and malformed, and no tentacular eyes were regenerated after 80 days (von Levetzow 1939). Also, in *Cryptocelis alba*, the eyes were not regenerated in the absence of the brain (Monti 1900); in *Leptoplana tremellaris*, cerebral eyes could be regenerated to some extent, though not in symmetrical clusters (Child 1904c). Both, tentacles and eyes, seem to be dependent on the presence of the brain during regeneration, at least to some degree (von Levetzow 1939), while all other body parts can regenerate normally in the absence of the brain.

Unexpectedly, in one experiment, double pharynges appeared in headless regenerates of *T. mediterranea*, reminiscent of *Schmidtea mediterranea* with overexpression of *beta-catenin* (Gurley et al. 2008). Already, Child noticed the appearance of a second pharynx in some headless regenerates of '*Cestoplana*', but in his case the old pharynx was still present and the new pharynx appeared posterior to the old one (Child 1905d). Other regeneration-deficient flatworms, that is flatworms that cannot regenerate the brain, are also able to regenerate major organs in the absence of the brain, e.g. the male genital organ in *Macrostomum lignano* (Egger et al. 2006).

A synopsis of polyclad regeneration

A new guide of polyclad regeneration is best drafted as a negative: polyclads cannot regenerate the whole brain. Adopting the classification in triclads (Sivickis 1930; Teshirogi et al. 1977), polyclads can be divided into four groups according to their regeneration capacity. Group I cannot regenerate anything but modestly sized peripheral parts, comprising at least the acotylean genus *Stylochus*, and possibly also containing *Discocelis tigrina* and the cotyleans

Stylostomum ellipse, *Maritigrella crozieri*, *Prosthiostomum siphunculus*, *Pseudoceros velutinus*, and *Yungia aurantiaca* (see all species of group I in Tables 1 and 2). All of the latter species require a reinvestigation to test their lack of regeneration capacity.

Group II polyclads can regenerate organs posterior to the brain, but either can not regenerate anteriorly or were not tested for anterior regeneration (all species of group II in Tables 1 and 2): here, *Cryptocelis alba*, an undetermined *Leptoplana* species, and *Prosthiostomum dohrni* are included, pending further studies.

The third group of polyclads (group III) can regenerate everything but their complete brain, and most of their organs can regenerate even in the absence of the brain (all species of group III in Tables 1 and 2). This is by far the largest group, and the acotyleans *Hylocelis californica*, *Notocomplana litoricola*, *Notocomplana saxicola*, *Leptoplana littoralis*, an undetermined leptoplanid, *Notoplana alcinoi*, and *Comoplana agilis*, as well as the cotyleans *Thysanozoon brocchi* and *Eurylepta cornuta*, are part of group III at this time.

Finally, group IV polyclads have been shown to regenerate also the anterior half of the brain. These are the acotyleans *Leptoplana tremellaris*, *Notocomplana humilis*, and the cotylean *Theama mediterranea* (see all species of group IV in Tables 1 and 2).

All listed groups are provisional, as more comprehensive studies may reveal new details, especially concerning species listed in groups II and III. At this time, we refrain from erecting more groups that include the regeneration capacity of the lateral brain half of *Leptoplana tremellaris* (Child 1904a, 1905a), or the regeneration of the whole brain in '*Cestoplana* sp.' (Child 1905b).

Stem cell distribution in control animals

In most studied free-living flatworms, the brain is the anterior border of proliferating cells, for example in the catenulid *Paracatenula galateia* (Dirks et al. 2012), the macrostomorphan *Macrostomum lignano* (Nimeth et al. 2007), the proseriate *Monocelis* sp. (Girstmair et al. 2014), and in several triclad species (Newmark and Alvarado 2000). In *Theama mediterranea*, the same basic pattern of stem cell distribution in the intact worm can be observed: a row of proliferating cells on each side of the animal, starting at the level of the brain and making a small loop before the tip of the tail (Fig. 1n). But in some polyclads, there are exceptions to this pattern: in adult *Prosthiostomum siphunculus*, S-phase neoblasts can be found in large numbers anterior to the brain, just behind the submarginal eyes at the anterior tip. Juveniles of *Pseudostylochus intermedius* also show proliferating cells anterior to the brain, and also anterior to all eyes (Egger et al. 2009b).

No proliferation takes place in the epidermis or in the pharynx (which is partially derived from ectoderm in polyclads (Boyer et al. 1998)) in *T. mediterranea*, or any other rhabditophoran flatworms studied so far (Egger et al. 2009b). Cell renewal of the epidermis (epidermal replacement) and of tissue anterior to the brain takes place through migration of cells that are not proliferating anymore (Egger et al. 2009b).

Stem cell dynamics in blastema proper and in organ primordia

In *Theama mediterranea*, the regeneration blastema at the posterior end harbours S-phase neoblasts throughout the observed period from 3 to 8 dpa (Fig. 8). Before 3 dpa, the blastema was too small to meaningfully show proliferation within the blastema, and after 8 dpa, the blastema is on the verge of having differentiated in large parts, although the proliferation is still more pronounced than in controls (Fig. 1n). A small posterior blastema was already noticed in Child's experiments, where he notes that 'the amount of posterior regeneration is never great' (Child 1905b).

In the polyclad *Notocomplana humilis*, the posterior blastema is more pronounced. In a recent study, proliferation in the posterior blastema was shown in histological sections, consistent with the results shown here (Okano et al. 2015). In *T. brocchi*, histological sections in 7 to 15 dpa posterior blastemas also showed neoblast stem cells within the blastema, although no proliferation marker like BrdU or EdU was available at the time (von Levetzow 1939). In that study, neoblasts were shown to be surrounding the pharynx primordium—a constellation also found in our pulse experiments (Figs. 7, S5). The same pattern (no cell proliferation in organ primordia) was observed in the regenerating genital primordium in *Macrostomum lignano* (Egger et al. 2009a) and the pharynx primordium in *Monocelis* sp. (Girstmair et al. 2014).

Tricladida is the best-studied group of free-living flatworms, and their regeneration blastema is mostly free of S-phase neoblasts. In Girardia, proliferation takes place in the so-called post-blastema (Saló and Baguñà 1984; Morita and Best 1984; Wenemoser and Reddien 2010). However, all other studied free-living flatworms show abundant S-phase neoblasts within the blastema, including the basally branching Catenulida and Macrostomorpha, and the—like Tricladida—euneoophoran Proseriata (Dirks et al. 2012; Egger et al. 2009a; Girstmair et al. 2014). The available data, supported by our findings in the polyclad *Theama*, suggest that a proliferation-free regeneration blastema is a derived character of Tricladida, and that the plesiomorphic condition in Platyhelminthes is proliferating cells within the blastema. In this respect, the closest relatives to Tricladida, i.e. Fecampiida and Prolecithophora (Laumer et al. 2015; Laumer and Giribet 2017), are interesting

candidates to determine if a blastema free of proliferation is an apomorphy of Tricladida, or a characteristic shared with other flatworm groups.

Conclusions

The only polyclad reported to be able to regenerate the complete brain was *Cestoplana sp.* (Child 1905a). After careful experimental and taxonomic reexamination, we conclude the polyclad studied by Child likely was *Theama mediterranea* and that it cannot regenerate the complete but only the anterior half of the brain.

This agrees with the regeneration capacity reported by a few other polyclads examined so far, but most polyclads cannot regenerate any part of the brain, and none can regenerate the whole brain (Tables 1 and 2). However, most organs can regenerate in the absence of the brain. Both polyclads and triclads show a large range of regeneration capacity between different species of the same order, but explanations for this phenomenon remain elusive.

In *T. mediterranea*, proliferating stem cells are absent in the epidermis and ectodermally derived tissues like the pharynx, as found in many other rhabditophoran flatworms. In regenerating *T. mediterranea*, S-phase cells are located in the regeneration blastema, similar to catenulids, macrostomorphans, and proseriates, but different from triclads, where the blastema is devoid of proliferation. These findings indicate that a proliferation-free blastema is a derived characteristic in flatworms.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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