

# Free-living flatworms under the knife: past and present

Bernhard Egger · Robert Gschwentner ·  
Reinhard Rieger

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**Abstract** Traditionally, regeneration research has been closely tied to flatworm research, as flatworms (Plathelminthes) were among the first animals where the phenomenon of regeneration was discovered. Since then, the main focus of flatworm regeneration research was on triclads, for which various phenomena were observed and a number of theories developed. However, free-living flatworms encompass a number of other taxa where regeneration was found to be possible. This review aims to display and to compare regeneration in all major free-living flatworm taxa, with special focus on a new player in the field of regeneration, *Macrostomum lignano* (Macrostomorpha). Findings on the regeneration capacity of this organism provide clues for links between regeneration and (post-)embryonic development, starvation, and asexual reproduction. The role of the nervous system and especially the brain for regeneration is discussed, and similarities as well as particularities in regeneration among free-living flatworms are pointed out.

**Keywords** Platyhelminthes · Planarian · Acoela · Rhabditophora · Catenulida

## Introduction

Regeneration research in flatworms has a long standing history, but was focussed on triclads since its beginning in 1774, when Pallas (Pallas 1774) described the regeneration of the triclads *Dendrocoelum lacteum* and *Bdellocephala*

*punctata* (from Brøndsted 1969). Since then, a vast amount of studies about regeneration in flatworms has appeared (for recent reviews, see Agata and Watanabe 1999; Sánchez Alvarado 2000, 2003, 2004, 2006; Saló and Baguña 2002; Newmark and Sánchez Alvarado 2002; Agata 2003; Agata et al. 2003; Reddien and Sánchez Alvarado 2004; Reuter and Kreshchenko 2004; Sánchez Alvarado and Kang 2005; Saló 2006), but many of the old questions and many of the most intriguing phenomena that have been discovered cannot be explained today. Why can some animals regenerate, while others cannot? How is the duplication of heads or tails (heteromorphoses) possible in adult organisms? What conditions are sufficient and what are necessary for successful regeneration? Is regeneration a side-product of asexual reproduction, or the other way round? Is regeneration recapitulating pathways used in embryonic and postembryonic development? How are stem cells controlled and directed in regeneration? Are stem cells in adult flatworms totipotent, and can they be likened to embryonic blastomeres? This review provides a brief synopsis on what is known about the regeneration capacity in free-living flatworms, and addresses open questions about regeneration, with special emphasis on an emerging model organism, *Macrostomum lignano* (Egger et al. 2006a,b).

A comparison of the regeneration capacity of the macrostomorph flatworm *M. lignano* with other free-living flatworms necessarily involves a look at their phylogenetic relationship. The Macrostomorpha belong to the largest taxon Rhabditophora, which also encompasses the Polycladida, Lecithoepitheliata, Prolecithophora, Proseriata, Bothrioplanida, Tricladida, Rhabdocoela, and the parasitic Neodermata (Ehlers 1985; Rieger 1996; Tyler et al. 2006). Macrostomorpha are considered to be the most basal taxon within the Rhabditophora due to a simple pharynx, entolecithal eggs, and the lack of vitellaria (Ehlers 1985). Referring to their small size (millimeter range), macro-

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B. Egger (✉) · R. Gschwentner · R. Rieger  
Ultrastructural Research and Evolutionary Biology,  
University of Innsbruck,  
Innsbruck, Austria  
e-mail: bernhard.egger@uibk.ac.at

stomorphans are members of the “microturbellarians”, a nonsystematic denomination. Polyclads and triclads are the only taxa belonging to the “macroturbellaria”, with forms generally in the centimeter range. Triclads, as more derived rhabditophorans, have vitellaria, a rather complicated embryonic development (e.g., Cardona et al. 2005) and an often very pronounced regeneration capacity (see Brøndsted 1969).

The status of the acoels

While many molecular (e.g., Bagaña and Riutort 2004a,b) and also some morphological studies (e.g., Reuter et al. 2001, summed up in Egger and Ishida 2005) have placed the acoels outside the Plathelminthes, a number of characteristics (apomorphies) speak in favor of acoels being flatworms (Ehlers 1985), most notably the stem cell system and the special mode of epidermal replacement (Smith et al. 1986; Tyler and Hooge 2004). In this review, acoels and nemertodermatids are considered members of the Plathelminthes.

What is a planarian?

In flatworm regeneration research, other groups than triclads are often neglected and can easily be overlooked. This may in part be due to an ambiguous use of the term “planarian”, which is most often referring merely to triclads (more specifically, to paludicolans, e.g., Agata 2003; Reddien and Sánchez Alvarado 2004), but is also commonly used as a synonym for all free-living flatworms (e.g., Bagaña 1998; Saló and Bagaña 2002) or even used interchangeably for free-living flatworms and triclads alike (e.g., Newmark and Sánchez Alvarado 2001). The term “freshwater planarian”, while somewhat more specific, is still ambiguous, as a number of nontriclade flatworms, e.g., *Stenostomum* and *Microstomum*, are freshwater dwellers as well. In publications dealing with several flatworm taxa, it is often unclear what the term planarian signifies.

It seems therefore desirable to agree on using less ambiguous denominations in scientific contexts, such as unequivocal scientific names, and to get rid of the term planarian.

### Overview of regeneration capacity in free-living flatworm groups

All major free-living flatworm taxa with their tentative general regeneration capacities are given in Fig. 1. Asexually reproducing forms are only known for acoels, catenulids, macrostomorphans, and triclads. Within free-living flatworms, either catenulids (Ehlers 1985) or acoelomorphs (acoels and nemertodermatids, Rieger 1996) are considered as the basal-most taxon. All other flatworms are

Regeneration capacity		
unknown		Nemertodermatida
excellent	asexual forms	Acoela
excellent	asexual forms	Catenulida
excellent	asexual forms	Macrostomorpha
good		Polycladida
poor		Lecithoepitheliata
good		Prolecithophora
good		Proseriata
excellent	asexual forms	Tricladida
good		Bothrioplanida
poor		Rhabdocoela

**Fig. 1** The regeneration capacity of the major free-living flatworm taxa. In all groups with asexual reproduction, species with a pronounced regeneration capacity can be found (green shades). Not all species in the listed taxa are necessarily showing the same regeneration capacity; only a broad classification of regeneration capacities is given. The major taxa were compiled after Ehlers (1985), Rieger (1996), and Tyler et al. (2006)

encompassed by the rhabditophorans, with macrostomorphans and polyclads at the base (Ehlers 1985; Rieger 1996).

#### Catenulida

The regeneration capacity of two catenulid genera, namely, *Stenostomum* and *Catenula*, has been extensively investigated. Probably linked with asexual reproduction (paratomy), excellent regeneration capacity has been reported after artificial amputation (Ritter and Congdon 1900; Child 1903a,b; Ruhl 1927a; van Cleave 1929; and Palmberg 1990 for *Stenostomum*; Moraczewski 1977 for *Catenula*). Regeneration from the so-called organ primordia was found to be possible for all organ systems.

#### Acoela

Based on experiments mainly with *Polychoerus caudatus* (Stevens and Boring 1905; Child 1907; Keil 1929) and *Paramecynostomum diversicolor* (Peebles 1913), Hyman notes that acoels “have limited powers of regeneration” (Hyman 1951, p 182). The studied species were not able to regenerate ganglia (brain) or the statocyst. Later studies with *Amphiscolops langerhansi* (Steinböck 1954, 1955, 1963a,b) and *Hofstenia giselae* (Steinböck 1966, 1967) illustrated a substantial regeneration capacity in these acoels, where *H. giselae* was found to even regenerate the

statocyst after amputation (Steinböck 1966). Several species can regenerate their eyes (Hanson 1967 for *A. langerhansi* and *Pseudohaplogonaria sutcliffei*, Yamasu 1991 for *Praesagittifera naikaiensis*, and Åkesson et al. 2001 for *Convolutriloba longifissura*).

Acoels are now known to show all main modes of asexual reproduction, that is architomy (e.g., *C. longifissura*, Åkesson et al. 2001), paratomy (e.g., *Paratomella unichaeta*, Ax and Schulz 1959), and budding (e.g., *C. retrogemma*, Hendelberg and Åkesson 1988). Architomy is characterized by fission before formation of new organs, paratomy by formation of new organs before fission. Budding refers to a special case of paratomy, where the axis of the new piece is not corresponding to the axis of the old piece—in *C. retrogemma*, the axis of the buds is tilted by 180° in relation to the main animal (Hendelberg and Åkesson 1988, 1991).

#### Nemertodermatida

No account of regeneration potential or asexual reproduction has been given for animals from this taxon.

#### Macrostomorpha

As already observed in catenulids and acoels, the regeneration capacity of macrostomorphans is closely associated with the mode of reproduction. Thus, in the asexually reproducing taxa *Microstomum lineare* and *Aleurina* (paratomy), the regeneration capacity is most pronounced and all organs can be regenerated (von Graff 1908; Ruhl 1927a; Palmberg 1986, 1990, 1991). Regeneration research with the exclusive sexually reproducing genus *Macrostomum* started with Ruhl (1927a), who found that *Macrostomum tuba* cannot regenerate the head (brain, eyes, and pharynx) and requires at least a quarter of the gut in the anterior piece to be able to regenerate posterior parts, including copulatory organs. More recently, Ladurner et al. (1997) noted that in *Macrostomum hystricinum marinum* and *Macrostomum pusillum* the anterior part can fully regenerate the posterior part, while the posterior part cannot regenerate the anterior part. Similar findings were reported for *M. lignano*: cut in midbody, only the anterior part was found to regenerate the missing part (Rieger et al. 1999). Salvenmoser et al. (2001) described the regeneration of the musculature in *M. lignano* after the removal of the posterior end. Using the same species, Egger et al. (2006a,b) characterized the regeneration capacity at various cutting levels. Anterior regeneration is possible if the animals are cut in front of the brain; posterior regeneration can be successful from cutting levels starting with the posterior end of the pharynx. A distinct regeneration blastema occurs in *M. lignano*, but not in *M. lineare*, where organ primordia are formed without conspicuous accumulation of neoblasts at the wound site (Palmberg 1986).

#### Polycladida

The best-studied polyclad genus regarding regeneration is *Leptoplana*. *Leptoplana alcynoe* (now *Letoplana alcinoi*) and *Leptoplana velutinus*, as well as *Thysanozoon brocchi* and *Cryptocelis alba*, were shown to regenerate posterior parts by Monti (1900a). Using *Leptoplana atomata*, Schultz (1901, 1902) observed the regeneration of the posterior part, including gonads and copulatory organs, whereas head and pharynx were not regenerated. Similar results were obtained with *Leptoplana tremellaris* and *L. alcinoi*, although the pharynx was found to regenerate here (Child 1904a–c, 1905a; von Levetzow 1939). *L. tremellaris* can regenerate both anterior and posterior parts if only about half the brain is removed by transversal amputation. At amputation levels posterior of the brain, no complete anterior regeneration is possible (Child 1904a–c). *Notoplana humilis* shows a comparable regeneration capacity (Ishida 1998). Besides more posterior parts, *T. brocchi* is also able to regenerate the main tentacles, gut, and pharynx (Monti 1900a; von Levetzow 1939). Studying regeneration in *Leptoplana saxicola*, *Pucelis litoricola*, and *Planocera californica*, Olmsted (1922a) reports that these species follow the “polyclad rule for regeneration”, that is, regeneration of all amputated parts (including eyes) is possible if the brain remains intact. Several studies were dedicated to find out whether polyclads can regenerate the brain (Morgan 1905 for *Leptoplana littoralis* and von Levetzow 1939 for *T. brocchi*), and how the behavior changes after excision of the brain (Morgan 1905; Olmsted 1922b; von Levetzow 1939; Koopowitz et al. 1975, 1976) or after severance of the main lateral nerve cords (Olmsted 1922b; Koopowitz et al. 1975, 1976). Severed nerve cords and brain halves can fuse and resume their functions (Olmsted 1922b; Faisst et al. 1980), but the brain is not regenerated. Full anterior regeneration, including the brain, after complete removal of the brain was only observed in *Cestoplana*, provided the amputation level is a short distance behind the brain (Child 1905b). All mentioned species, except *T. brocchi*, are members of the taxon Acotylea (Tyler et al. 2006). From what is known, both Cotylea and Acotylea seem to share a comparable regeneration capacity.

#### Lecithoepitheliata

Only scarce reports about regeneration are available for this neoophoran taxon. For *Prorhynchus stagnalis*, Ruhl (1927a) remarks that no regeneration capacity whatsoever could be detected at anterior, posterior, and midbody cutting levels. The posterior pieces survived for 30 days. When cutting *Geocentrophora baltica* a short distance behind the pharynx, no regeneration could be observed (Steinböck 1927). All pieces died at the latest after 17 days.

A closer examination of the regeneration capacity of *Geocentrophora sphyrocephala* revealed that these animals can approximately rebuild the mouth opening anteriorly in 3–5 weeks but cannot regenerate the brain. Both parts die after 40 days if the animals are cut just behind the pharynx. Cut in midbody or more posteriorly, the anterior part can regenerate the tail with adhesive organs in 2–3 weeks, although the animals were never observed to reach the original body length. The posterior part dies without regeneration after 1 day (Hagleithner 1946).

#### Prolecithophora

According to Pechlaner (1957), *Plagiostomum lemani* is reported to have a low regeneration capacity (Keller 1894), while *Plagiostomum girardi* can regenerate all amputated parts (Monti 1900b).

#### Proseriata

von Graff (1882) describes amputation experiments with *Monocelis fusca*, where posterior pieces resumed movement after 3–5 days. In *M. fusca*, *Monocelis lineata* and other monocelidids (*Coelogygnopora*, *Archilopsis*), and also in otoplanids (*Itaspiella*, *Bothriomolus*), Giesa (1966) observed posterior regeneration of parts containing the brain in 3–4 days. Even pieces amputated just behind the brain can regenerate. The brain does not regenerate, though. In *Otomesostoma auditivum*, Pechlaner (1957) found promising regeneration potential, severely limited by a short life span of 9–10 months and low living temperatures (about 4°C). Gut, part of the gonads, and sensory organs can regenerate. First signs of pharynx, eye, and brain regeneration were observed after 9–15 weeks, but the animals died before regeneration was complete. The statocyst was not regenerated.

#### Bothrioplanida

While no extensive regeneration studies have been undertaken, Sekera (1911) found that *Bothrioplana semperi* is able to regenerate the pharynx posteriorly.

#### Tricladida

Dalyell (1814) noted that *Planaria nigra* (today: *Polycelis nigra*) could “almost be called immortal under the edge of the knife” (in Brøndsted 1969). However, not all triclad species are equally powerful regarding regeneration. Šivickis (1930) distinguished five groups of triclads according to their ability to regenerate a head. This classification was subsequently refined into eight types by Teshirogi et al. (1977). For the *Phagocata velata* type, the

*Dugesia dorotocephala* type, the *Polycelis auriculata* type, and the *Dendrocoelopsis lactea* type 2 (Sapporo population), regeneration of a head is possible from any part of the body, but with varying probability of success. Animals of the *Dendrocoelopsis lactea* type 1 (Kuroishi population) fail to regenerate a head from tail pieces up to the male gonopore. The *Dendrocoelopsis ezensis* type and the *Dendrocoelum lacteum* type 1 are very similar in that they are generally only able to regenerate a head if amputated anterior to the pharynx, whereas the *Bdelloura* type (e.g., *Bdelloura candida*) completely lacks the ability to regenerate a head. While a vast amount of literature on regeneration in triclads is available, recent publications are not so much concerned with studying the regeneration capacities of different triclads, but more with unraveling cellular and molecular mechanisms of regeneration in only a few model triclads, such as *Dugesia japonica* and *Schmidtea mediterranea*.

#### Rhabdocoela

The typhloplanoid flatworm *Mesostoma ehrenbergi* cannot regenerate any organs, only approximately restore the original shape posteriorly (Steinmann and Bresslau 1913). Regeneration experiments with *Mesostoma productum*, *Mesostoma lingua*, and *Mesostoma punctatum* revealed that anterior regeneration of parts in front of the brain is possible, although often done imperfectly. The tail can regenerate; the more posterior the cut is made, the better is the regeneration. Brain and pharynx cannot be regenerated (Fulinski 1922). In *Phaenocora unipunctata* and *Phaenocora megalops* (also typhloplanoids), no regeneration capacity was found (Fulinski 1922). For the the dalyelliid *Dalyellia millportiana* and the kalyptorhynch *Gyratrix hermaphroditus*, Ruhl (1927a) remarks curtly that all animals died within 1 day after amputation and no regeneration being observable. *Dalyellia viridis* and *Typhloplana viridata* survive the amputation of the caudal-most part of the tail, but show no signs of regeneration, not even of the adhesive organs. In *Rhynchomesostoma rostratum*, another typhloplanoid, the amputated tail tip is regenerated, or at least a tail-like shape is restored (Hein 1928).

### Comparison of the regeneration capacity between free-living flatworm taxa

While the experimental evidence for some groups is still patchy, we find three main types of regeneration capacity in free-living flatworms. Some taxa cannot regenerate at all, such as most rhabdocoels and some lecithoepitheliates. On the other end of the scope are species that can regenerate all organs, such as the acoel *Convolutriloba*, the catenulid *Stenostomum*, the macrostomorph *Microstomum*, the

polyclad *Cestoplana*, and many triclads. Most taxa however lie in between these extremes, lacking the ability to regenerate brain, eyes, pharynx, or statocyst (many acoels), but are able to regenerate gonads, copulatory organs, sucker, duo-gland adhesive systems, gut, and the anterior-most part in front of the brain.

The regeneration capacity of *M. lignano* can be likened most closely to triclads of the *Bdelloura* group, which cannot regenerate a head (brain or eyes). Most polyclads, although often able to regenerate a pharynx, are also roughly comparable to *Macrostomum* regarding their regeneration capacity. Members of other diverse taxa like lecitheopheliates and rhabdocoels are bearing resemblance with regeneration in *Macrostomum* as well. Such similarities can be found crossing the boundaries of the major flatworm taxa, making the regeneration capacity a feature of no immediate phylogenetic value. Close relatives like *Microstomum* and *Alaurina* (both macrostomids) surpass *Macrostomum*'s regeneration capacity by far.

### Asexual reproduction and regeneration

Not surprisingly, the ability to regenerate is often linked to asexual reproduction (Fig. 1, Brøndsted 1969; Sánchez Alvarado 2000). In taxa lacking asexual reproduction, the regeneration capacity is generally less pronounced than in taxa with asexual reproduction, where regeneration is necessarily involved (Brøndsted 1969; Reuter and Kreshchenko 2004). As described in the section “Acoela”, in architomy, regeneration events follow fission, while in paratomy and budding, the term “pregeneration” seems to be more accurate, as organs are duplicated in the presence of the old organs in the new animal (zooid) before fission occurs. The notable difference between regeneration and pregeneration is the presence of a brain in the latter, which is regarded as a decisive organ for regeneration in many species (Henley 1974; Kreshchenko et al. 2001). It is interesting to note that the catenulid *Stenostomum leucops*, reproducing asexually by paratomy, often fails to regenerate a head if amputated artificially (van Cleave 1929). This finding hints at an actual difference between paratomy and architomy regarding the influence on regeneration capacity.

Asexual reproduction in flatworms occurs in more basal taxa (Acoela, Catenulida, and Macrostomorpha) as well as in more derived taxa (Tricladida and Neodermata). Triclads, like acoels, show paratomy (*Dugesia fissipara* and *Dugesia paramensis*, Hyman 1951) as well as architomy (e.g., *Dugesia tahitiensis*, Peter et al. 2001). Just like regeneration capacity, asexual reproduction is too dispersed among diverse taxa to provide insight into phylogenetic relationships.

### Why can some flatworm species regenerate while others cannot?

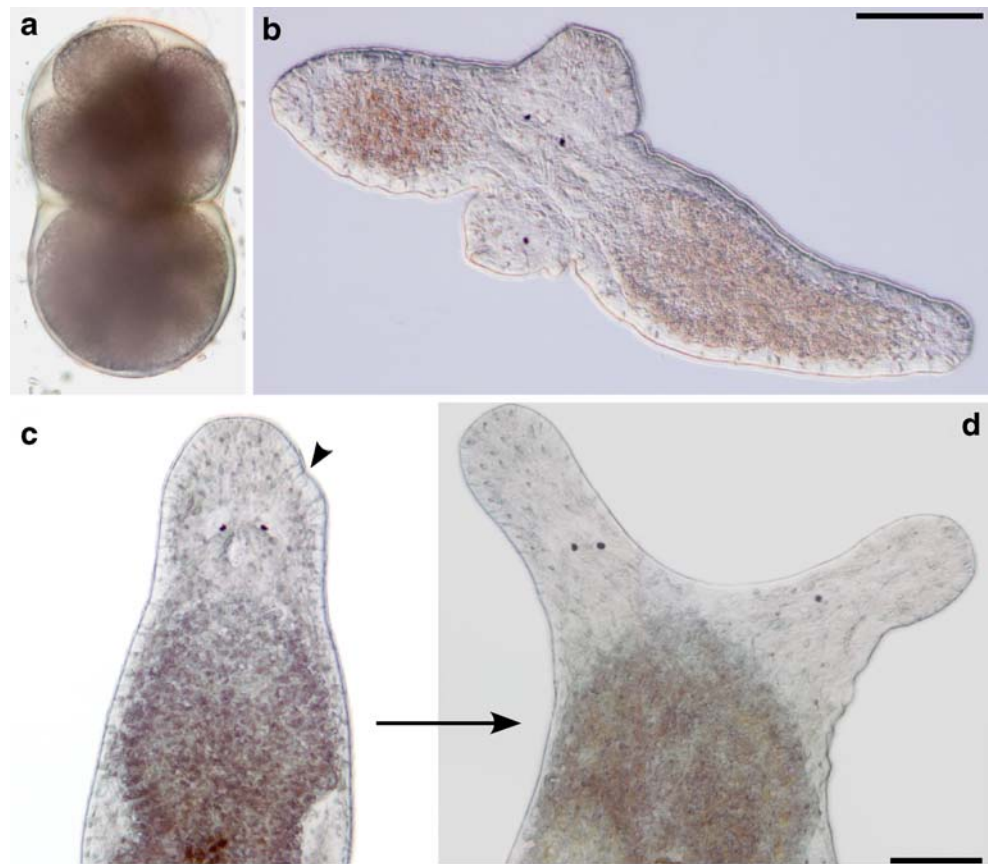
If a profound regeneration capacity is a plesiomorphic feature, there might have been a selection pressure against regeneration during evolution (Goss 1991; in Sánchez Alvarado 2000). Why would such a useful feature be reduced or abolished? Because regeneration is dangerous, as a plethora of evidence of double-headed or double-tailed flatworms testify. Such aberrant forms are easily produced in species that can regenerate (see section “Heteromorphoses”), but are not known from flatworms lacking regeneration capabilities. Other than as a side effect of asexual reproduction, the ability to regenerate is seemingly not useful enough to outweigh the inherent dangers. What are the chances that a predator spares a tail fragment that can regenerate to a complete animal in a couple of days if left alone? What are the chances that a disease only affects a part of the animal, but spares the rest to readily regenerate the damaged organs? A totipotent stem cell system out of control might be too high a risk for comparably little reward. This could be the reason why regeneration capabilities decreased in species that no longer propagate through asexual reproduction.

In this light, it seems more likely that in evolutionary terms, asexual reproduction is a primitive and not a derived feature (Rieger 1986; Gremigni 1992; Reuter and Kreshchenko 2004), and that regeneration capability results from asexual reproduction as an added bonus. Regeneration can be seen as a form of asexual reproduction, only triggered by different stimuli.

What is the decisive factor that determines the regeneration capacity of a species? Citing regeneration experiments with *Dendrocoelum* and *Bdellocephala*, Brøndsted (1969) comes to the conclusion “that the posterior part of the body in these two species simply lacks the potential to respond to head inducing substances”. This may also hold true for *M. lignano*, considering that in transversal amputations a head was never regenerated, but in longitudinal incisions a second head—in the presence of the original head—could be formed (Fig. 2c,d, Egger et al. 2006a,b). This experiment suggests that the ability to induce head formation, although present in adult *M. lignano*, is restricted to the head region only. Serotonin and possibly other substances related to the brain or nervous system might be key factors deciding on the regeneration capacity of flatworms.

It is interesting to note that double-headed specimens of *M. lignano* can sometimes be observed in untreated culture dishes. These aberrant forms might rather be the result of twin embryos (Fig. 2a) that have grown together during embryonic development, than adults that were accidentally incised longitudinally (Sekera 1911; Steinmann and Bresslau 1913; Seilem-Aspang 1958).

**Fig. 2** Heteromorphoses resulting from embryogenesis and regeneration. **a** Twin embryo of *M. lignano*, 8-cell stage. The two embryos are not separated by a dividing wall. In some cases, such embryos may grow to siamese twins. Diameter of individual embryos is about 150  $\mu\text{m}$ . **b** Different animal than **a** found in culture dishes. The juvenile animals are connected at the level of the pharynx, possibly the result of two embryos growing together. **c** Adult *M. lignano*, 1 week after longitudinal incision (arrowhead). Only a small indentation is visible. **d** Same specimen as in **c** 29 days after longitudinal incision. A second head with brain, a single eye, and pharynx has emerged. Scale bars are 100  $\mu\text{m}$ . **c**, **d** Same scale bar



### Ovaries restricting regeneration?

The possible evolutionary origin and function of regeneration aside, what genetical, physiological, developmental, or environmental factors are responsible to determine to what extent an organism can regenerate? Even close relatives may differ vastly in regeneration capacity (e.g., *Microstomum*–*Macrostomum*), and also the same species in different developmental stages. *Stenostomum* alternates seasonally between asexual and sexual reproduction (protandric hermaphroditism). Asexual and male specimens of *Stenostomum grande* can regenerate all organs, but lose the ability to regenerate a head after the appearance of ovaries (van Cleave 1929). While the presence of ovaries and the loss of regeneration capacity might be coincidental, it appears possible that in *S. grande* ovaries are producing substances that inhibit regeneration. Could sexualization be directly affecting a general decrease of regeneration potential? At present, the molecular nature of such an inhibitory substance remains unclear. *Vasa*-related genes are expressed strongly both in gonads and in the early blastema in *D. japonica* and in *M. lignano* (Shibata et al. 1999; Pfister and Ladurner 2005), making an inhibitory function of *vasa* during regeneration improbable, at least in these two species. Also, contrary to the findings in *Stenostomum*, several triclad are known to regenerate all organs in

the presence of gonads (e.g., *S. mediterranea*, Sánchez Alvarado 2003). Two possibilities may explain differences in the regeneration capacity in the presence of ovaries: either the ovaries in *Stenostomum* are producing a unique inhibitory substance that is not synthesized in *Schmidtea*, or *Schmidtea* is able to counteract the effects of such an inhibitory agent.

### Regeneration in juveniles

If gonads, specifically ovaries, are indeed hindering regeneration in some species, then sexually immature juveniles could possibly be more potent regenerates than adults. In the rhabdocoel *Mesostoma*, young animals (the author does not specify their age) are reported to regenerate better than adults (Fulinski 1922). Contrastingly, in the acoel species *H. giselae*, no regeneration even of large juveniles (2.5 mm long) takes place (Steinböck 1967).

In most cases, however, juveniles regenerate as well (or bad) as adults. An explanation is that gene expression for the building of gonads is already occurring in immature animals, thus diminishing possible differences in the regeneration capacity between juveniles and adults. Regeneration in juvenile triclad has been studied in *B. punctata* by Brøndsted, who found that animals recently hatched

from cocoons were able to regenerate the head at the same rate as mature adults (Brøndsted 1942, 1969). Pechlaner (1957) studied the regeneration of freshly hatched animals, juveniles and mature adults in the proseriate *O. auditivum*. He did not observe significant differences in the regeneration capacity between these age groups. Neither juvenile nor adult dalyellids were found to regenerate (Hein 1928). In the lecithoepitheliate *G. sphyrocephala*, the regeneration capacity of juveniles corresponds to that of adults (Hagleithner 1946). The results for *M. lignano* support these findings and show that even very young animals (1 day after hatching) are capable of regeneration, indicating that the neoblast stem cell system is already present and functional as found in adults. Accordingly, BrdU labeling of 1-day-old hatchlings revealed a similar pattern of S-phase cell distribution as in adults, with the majority of labeled cells along the sides of the animal, and no labeled cells in the rostrum. Just like adults, juveniles were not able to regenerate a head (Egger et al. 2006a,b).

### Similarities between postembryonic development and regeneration

However similar, regeneration has some characteristics not shared with postembryonic development. Unlike postembryonic development, regeneration in flatworms is triggered by a disruptive event like fission or amputation (but not by chemical destruction of tissue, burning, or high voltage; see Brøndsted 1969). As in other flatworms, in *M. lignano*, the wound is soon closed after amputation by contraction of the ring musculature minimizing the wound surface, and by flattening of the surrounding epidermal cells (Salvenmoser et al. 2001). In the next days, undifferentiated cells (neoblasts) accumulate at the wound site, forming a distinct blastema, covered by new and old epidermal cells. Subsequently, differentiation of microorgans and organs becomes prevalent within the blastema, superseding the blastema proper (Egger et al. 2005, 2006a,b). Epidermal cells are not undergoing mitoses, as new epidermal cells and blastema cells are derived from neoblasts located in the mesodermal space. Different to triclads, neoblasts are proliferating also within the blastema, not only at the blastema border (Egger et al. 2005). While wound closure and blastema formation are regeneration-specific events, similarities between regeneration and postembryonic development become prevalent starting with differentiation in the (former) blastema. In extreme cases, where only a small piece remains for regeneration (e.g., in pharynx-level regenerates, Egger et al. 2006a,b), resemblance with postembryonic development is particularly striking (Fig. 3). The remaining organs in these regenerates undergo extensive morphallactic changes

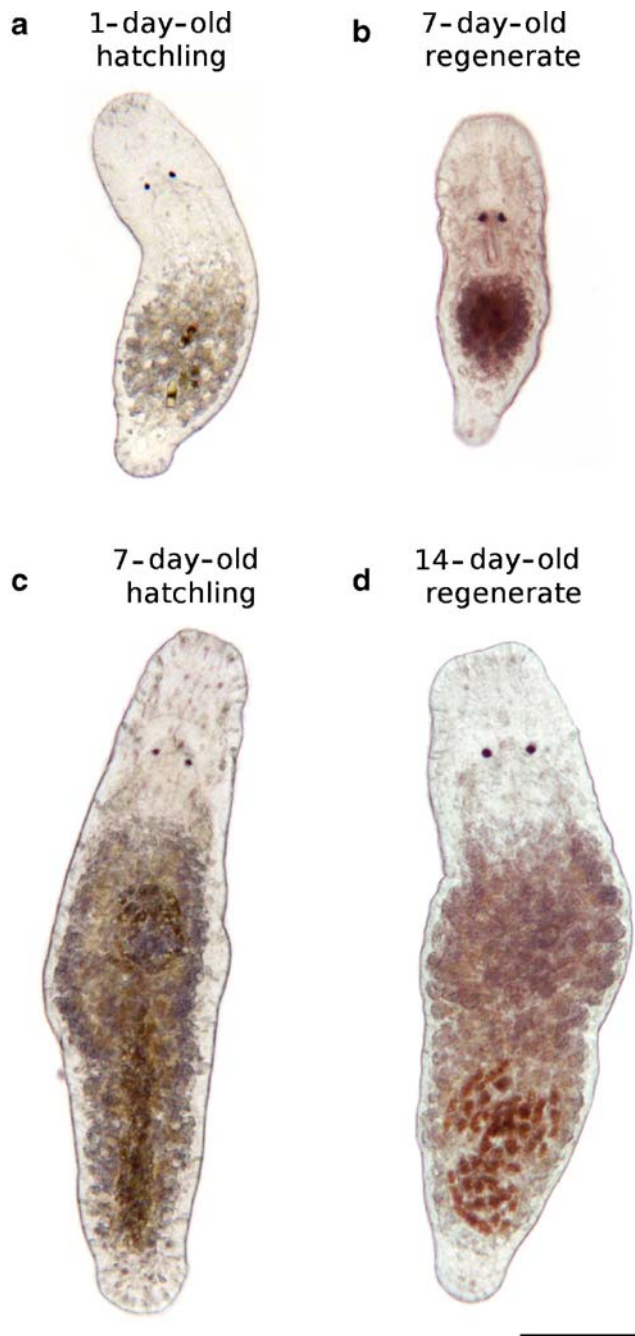
to accommodate to the new body proportions (Fig. 3n–o in Egger et al. 2006a).

One week after amputation, pharynx-level regenerates of *M. lignano* are confronted with similar challenges as freshly hatched juveniles: both have to build gonads and the male and female genital organs, grow a full set of duo-gland adhesive glands, and increase their size by five or six times (Figs. 3a,b, and 4). It seems plausible, that after wound healing and restoration of the basic functionality of the gut, pharynx-level regenerates are making use of similar or same developmental pathways that are used in hatchlings (Fig. 4) (cooption, Sánchez Alvarado 2000). This view is supported by the ability of hatchlings and juveniles to regenerate just like adults, and by the similarity of the stem cell system in hatchlings, juveniles, and adults (Ladurner et al. 2000; Egger et al. 2006a,b). *Vasa* gene expression is similar in hatchlings and in gonad-recovering regenerates as well (Pfister and Ladurner 2005). Also, the time needed for developing to a full-grown adult in *M. lignano* is about the same for hatchlings (Morris et al. 2004) and 1-week-old pharynx-level regenerates, that is about 2 weeks (Fig. 3 and Egger et al. 2006a,b). In the proseriate *M. fusca*, the regeneration of small anterior pieces also goes through stages similar to postembryonic development (Giesa 1966), which is observed in triclads as well (Baguña 1998). Starved adults, reducing their size and breaking down the gonads (Nimeth et al. 2004), are also likely recapitulating their postembryonic development while growing back to full size (Fig. 4). Not only is regeneration akin to postembryonic development, amputated animals can even be rejuvenated by regeneration (Fig. 4 and Haranghy and Balázs 1964 for triclads, Egger et al. 2006a,b for *M. lignano*). While untreated animals were found to live for about 10 months in culture conditions, they were outlived by animals amputated 45 times over a period of 20 months, effectively doubling their life span so far (Egger et al. 2006a,b; Egger, unpublished observations). At the time of writing, many of these repeatedly amputated animals are still alive.

A fountain-of-youth effect through starvation was shown to occur in triclads (Haranghy and Balázs 1964), but was not yet proven for *M. lignano* (Fig. 4).

### Embryonic development and regeneration

Early embryogenesis is vastly different between diverse free-living flatworm taxa (for recent publications, see Jondelius 2004 for nemertodermatids; Ramachandra et al. 2002 for acoels; Morris et al. 2004 for macrostomorphans, Younossi-Hartenstein and Hartenstein 2000 for polyclads, Cardona et al. 2005 for triclads, and Younossi-Hartenstein and Hartenstein 2001 for rhabdocoels). Even comparably



**Fig. 3** Comparison of *M. lignano* hatchlings with posterior regenerates amputated at the pharynx level. **b** and **d** are depicting the same individual. **a** Hatchling up to 24 h old. **b** Pharynx level regenerate 7 days after amputation. **c** 7-day-old hatchling. **d** Pharynx level regenerate 14 days after amputation. Scale bar for all subpanels is 100  $\mu$ m

close relatives show significant differences in their early embryonic development. Lacking vitellaria and therefore producing entolecithal eggs, Macrostomorpha and Polycladida are the only archoophoran Rhabditophora, placing them at the basal-most positions in this largest flatworm taxon (Ehlers 1985; Rieger 1996). Nonetheless, their early embryonic development starts to deviate after about the

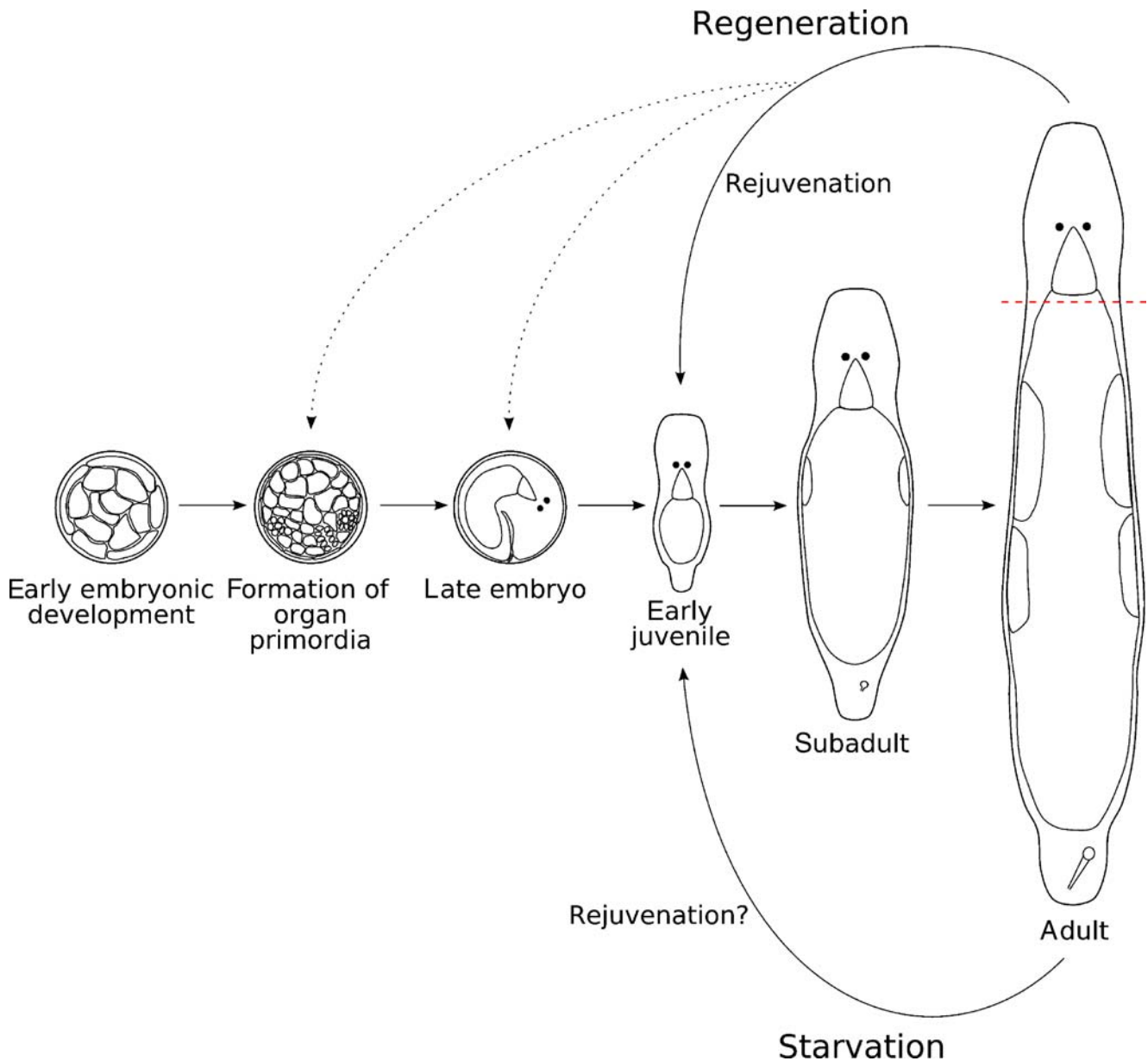
third cleavage (Fig. 5). If developmental pathways from the embryonic development are reused in regeneration, it is most likely from the formation of organ primordia and later stages (Fig. 4). Cardona et al. (2005) point out the similarities between embryogenesis and early regeneration in triclad concerning the building of a provisional epidermis and the development of muscles and nerves, suggesting that not only postembryonic, but also embryonic developmental pathways are utilized during regeneration, a viewpoint shared with Vannini (1966). A comparison of gene expression patterns between regenerates (see Reddien et al. 2005 for triclad), late embryos and hatchlings seem to be a worthwhile task.

Early blastomere ablation in polyclads has revealed a determinative development as in other spiralian (Boyer 1986), the fate of blastomeres depending on micromere–macromere interaction (Boyer 1989). Polyclads do not have an invariant cell lineage, however (Boyer 1992). Polyclad embryos can be obtained devoid of an eggshell (Fig. 5a) and still develop normally in a culture dish treated with antibiotics. Regeneration experiments with polyclad embryos may thus be relatively easy to carry out and could provide additional clues about the requirements for regeneration in flatworm embryos. Also, the regeneration capacity of polyclad larvae, as compared to hatchlings of directly developing polyclads, would be interesting to know. A connection between regulation in early embryonic development and regeneration capacity does not seem to exist. This notion is supported by regeneration experiments with embryos of the triclad *P. nigra*. Regeneration does not take place before organ systems, such as the nervous system, reach a certain degree of differentiation in the embryo, so that stage five embryos are unable to regenerate, while stage six embryos with a more differentiated nervous system readily regenerate lost parts (Le Moigne 1966). This author held that the nervous system plays a critical role in the formation of a blastema during regeneration.

### Nervous system and role of the brain for regeneration

The nervous system, especially the brain, has long been considered to play a key role in regeneration (Kreshchenko et al. 2001). In many free-living flatworms, posterior parts can be regenerated, while the brain cannot. In anterior regenerates of *M. lignano* missing the brain, a blastema-like structure was observed, which was unable to give rise to all missing organs. The more posterior the animals were amputated, the less pronounced was this abortive anterior blastema (Egger et al. 2006a,b). In *Macrostomum*, are the brain and the nerve cell clusters associated with the pharynx (Fig. 6) required to release factors needed for the regeneration of brain, eyes, and pharynx? The study of





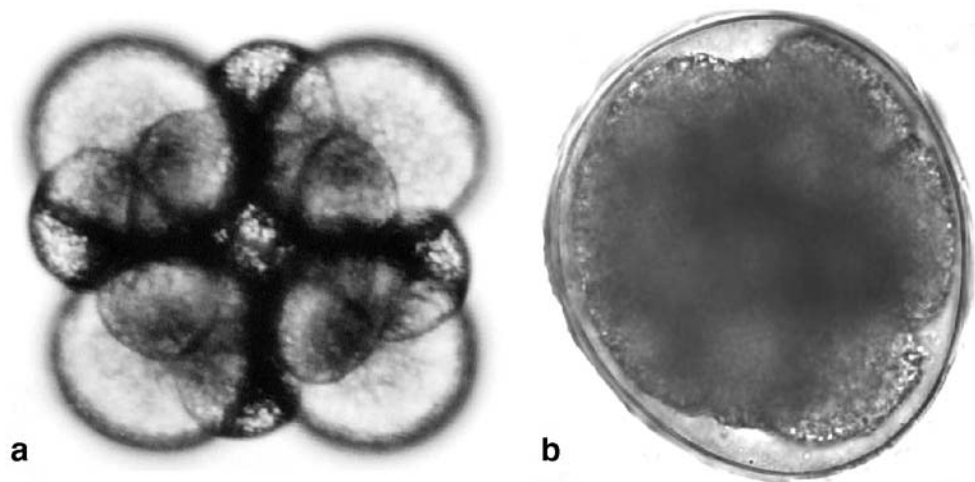
**Fig. 4** Similarities between (post-)embryonic development, regeneration, and starvation considering as example *M. lignano*. Successive developmental stages from *left to right*, from embryonic to postembryonic development (formation of gonads and genital organs) until adulthood. **Regeneration** If an adult animal is amputated just posterior of the pharynx, the anterior piece will pass stages similar to early juveniles and subadults before regeneration to a normal adult is completed. The same animal can be amputated repeatedly, each time seemingly repeating parts of its postembryonic development. Repeated regeneration was also shown to have a rejuvenation effect. A connection between the formation of organ

gene expression in head-amputated regenerates of the triclads *D. japonica* and *S. mediterranea* revealed necessary genes for regeneration of the brain (Cebrià et al. 2002a; Koinuma et al. 2003; Cebrià and Newmark 2005). Also, with *nou-darake* a factor was identified that restricts brain tissue to the head region of *D. japonica*. If *nou-darake* is

primordia during embryonic development and the organ differentiation in regenerating animals is possible, but has not been shown yet for *M. lignano*. Some developmental pathways are likely shared between late embryos and regenerates as well, e.g., the initial building of microorgans like the duo-gland adhesive systems. Both in embryos and posterior regenerates, these organs are built from scratch. **Starvation** Animals starved for 30 days or longer break down their gonads and genital organs shrink, but can recover and regain sexual prowess after feeding. Starvation leading to an extended life span was shown for triclads and is also likely to occur in *M. lignano*

interrupted by RNA interference, brains appear in all body regions (Cebrià et al. 2002b). In species that are not able to regenerate a brain, *nou-darake* or a similar factor might be expressed not only in the brain region but also throughout the body, thus preventing the formation of a new brain even if the old brain was lost.

**Fig. 5** Early embryonic development of two basal rhabdiphorans. **a** The 12-cell stage of the polyclad *Pseudostylochus intermedius*. Egg without egg-shell. **b** 12-cell stage of the macrostomorphan *M. lignano*. Different to the clear spiral cleavage of polyclads, *M. lignano* forms the so-called hull cells after the third cleavage to enclose the rest of the embryo. The diameter of the embryos is about 150  $\mu\text{m}$



In *M. hystricinum marinum* and *M. pusillum*, large clusters of serotonergic cells have been found besides the posterior part of the pharynx (Ladurner et al. 1997). These results were corroborated for *M. lignano* by staining of the serotonergic and the GYIRFamideergic nervous system (Fig. 6). Amputees cut in the middle of the pharynx lose this cluster of nerve cells, as well as a large part of the pharynx, which is known to be a critical structure for regeneration in some triclads (Brøndsted 1969) and polyclads (Ishida 1998). Ladurner et al. (1997) held that the distribution of serotonergic nerve cells is correlated with the regeneration capacity. In species where serotonergic neurons are restricted to the head area, the posterior part of the animals cannot regenerate the head. In the asexually reproducing species *M. lineare* and *S. leucops*, serotonergic nerve cells are found over the whole length of the main nerve cords.

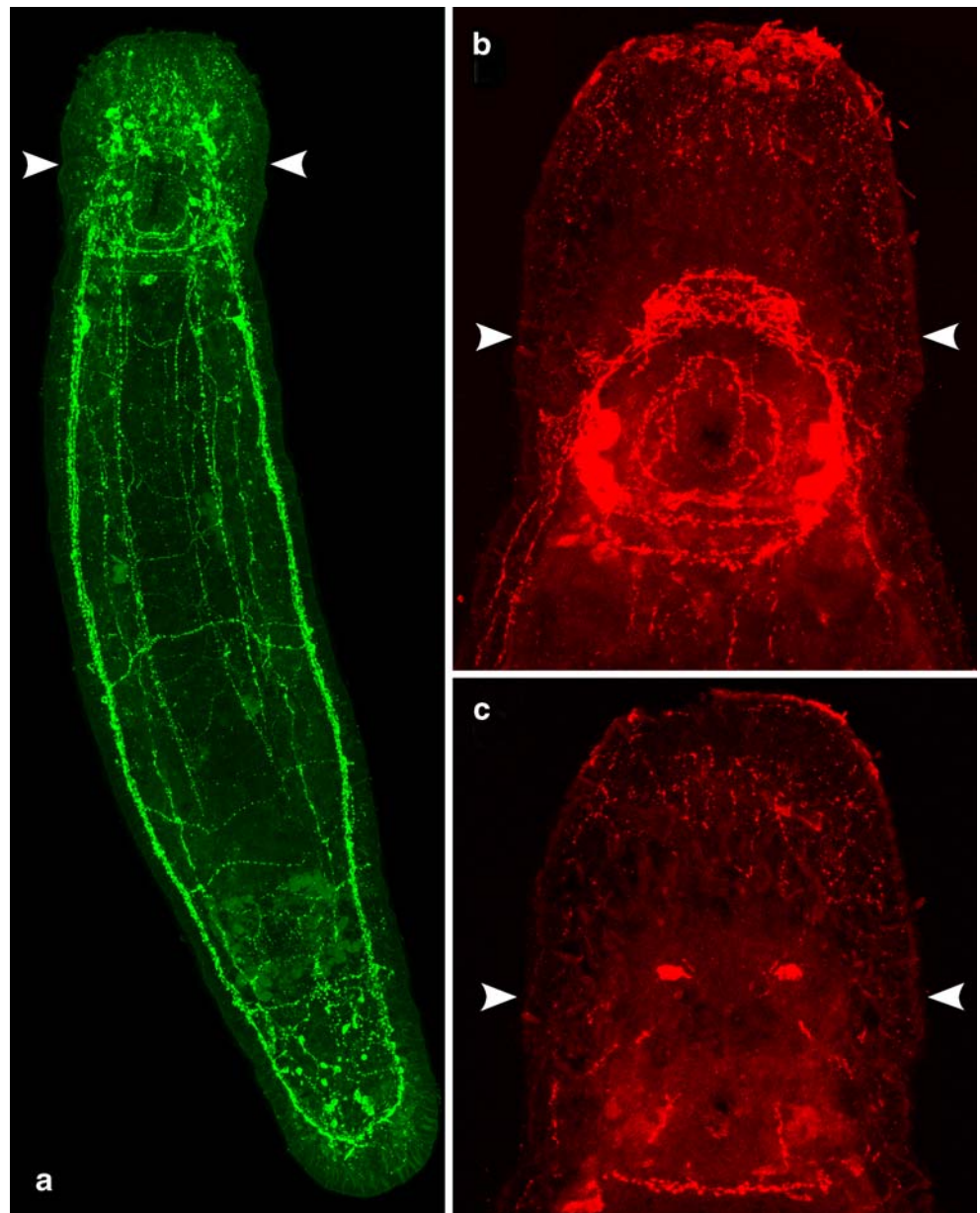
The inability of *M. lignano* to regenerate eyes after transversal amputation or oblique amputation between the eyes, is possibly linked with a severe brain damage caused by these amputations, and the loss of strongly immunoreactive cells in front of the eyes (Fig. 6c). In two cases of longitudinal incisions, lost eyes were regenerated or supernumerary eyes were built—but in these cases, the brain was only slightly injured. In a series of experiments, Lender (1950, 1951a–c) and Wolff and Lender (1950a,b) stress the importance of the head ganglia for the regeneration of eyes in *P. nigra*. If the brain was repeatedly excised or X-irradiated, no regeneration of eyes took place: an intact brain was obviously required to induce eye regeneration. These results were corroborated for *Dugesia* (now *Schmidtea*) *lugubris* by Török (1958). However, *L. littoralis*, *L. saxicola*, and *P. litoricola* can regenerate eyes even if the brain was excised (Morgan 1905; Olmsted 1922a).

Vannini (1965) observed in *S. lugubris* and *P. nigra* that the testes do not regenerate in the absence of a brain. Ghirardelli (1965) found that if a two-headed *Dugesia* was decapitated on one side, the testes on the same side

degenerated. Contrastingly, the gonads in *M. lignano* are viable for an extended period of time after decapitation or oblique amputation between the eyes, and are only reduced if the pharynx is lost as well, causing malnourishment and starvation (Egger et al. 2006a,b). This may be an indication that the building of the gonads is not under the primary control of the brain in *M. lignano*, just like the tail plate can be built without the presence of a head. The regeneration of a tail in headless regenerates is possible in *M. lignano* as well as in triclads of the *Dendrocoelum* group (Brøndsted 1939, 1969), in the polyclads *L. littoralis*, *L. saxicola*, and *P. litoricola* (Morgan 1905; Olmsted 1922a), and also in the rhabdocoel *Mesostoma* (Fulinski 1922). Therefore, the head in all of these organisms is not required to control the regeneration of the tail. In *Cestoplana* and *T. brocchi*, the pharynx can regenerate in the absence of the brain (Child 1905c; von Levetzow 1939); in *Cestoplana* often a secondary pharynx is built in the presence of the old pharynx (Child 1905c). Also, the regulation of tissue breakdown (e.g., gonads) and body size reduction during starvation is possible in decapitated pieces, so the brain is not responsible for this regulation as well. Headless *M. lignano* with intact pharynx are able to feed, in accordance with the polyclads *Notoplana acticola* and *Planocera gilchristi* (Koopowitz et al. 1976). These animals are able to identify and devour food in the absence of a brain, while *Enchiridium punctatum*, a polyclad possessing a tubular instead of a plicate pharynx, cannot feed without the brain (Koopowitz et al. 1976). Similarly, some triclads, also equipped with a tubular pharynx, are not able to feed if the brain was amputated (Hyman 1951). Brainless *M. ehrenbergi* cannot feed but can move normally (Steinmann and Bresslau 1913), whereas brainless *Phaenocera* is reported to behave similarly to normal animals (Fulinski 1922). *G. sphyrocephala*, different to *M. lignano*, cannot adhere to the substrate after decerebration (Hagleithner 1946).

The main function of the brain in *M. lignano* is probably to be found in directed motion (e.g., light avoidance,

**Fig. 6** Immunocytochemical stainings of the nervous system of *M. lignano*. Confocal images. **a** GYIRFamidergic immunoreactivity. Clusters of immunoreactive cells are found lateral to the pharynx and in front of the eyes. **b, c** Serotonergic immunoreactivity in the head region. **b** Full stack of confocal images. Note the stained cell clusters lateral to the pharynx and in front of the eyes. **c** Subset of image stack in **b**. In this focal plane, stained cells anterior to the eyes become apparent. *Arrowheads* denote the level of the eyes



predator avoidance; see also Koopowitz et al. 1976 and literature therein), satiation reflex (Koopowitz et al. 1976), mating behavior (Scharer et al. 2004), and chemical analysis of the medium.

### The totipotency of neoblasts

Pharynx-level regenerates of *M. lignano* have shown that the regeneration of gonads is possible after removal of all gonadal tissue. The viability of the regenerated gonads has been demonstrated by breeding experiments with fully regenerated animals. Besides the gonads, also the gut and the copulatory organs have been rebuilt de novo in pharynx-level regenerates (Egger et al. 2006a,b). In similar

experiments, Morgan (1902) removed all gonads in *Schmidtea lugubris*, and the animals regenerated to fully functional sexual organisms. It is still possible that the remaining neoblasts in the amputee are only pluripotent and not totipotent, different types of neoblasts being competent for different (groups of) tissues. A convincing experiment to prove the totipotency of neoblasts would be the injection of a single neoblast into a lethally X-irradiated animal that is to be amputated some weeks after the injection. In case the animal survives, and is able to restore all removed organs, then either redifferentiation of cells would have taken place, or (at least some) adult neoblasts would be totipotent. A similar experiment, with the injection of 20,000–24,000 cells enriched in neoblasts (30–88% neoblasts) into X-irradiated *S. mediterranea*, was performed by

Baguña et al. (1989). This experiment was not replicated in the literature, but the results made highly probable that neoblasts as a whole are totipotent in routine cell turnover, leaving open the questions if this holds also true for regeneration and if this totipotency applies to one single cell type or to several different types of stem cells.

Are neoblasts in species with limited regeneration potential not totipotent, or are they just not receiving the right stimulus or guidance for regeneration? Supporting the latter notion is the ability of *Macrostomum* to duplicate heads after longitudinal incision (Fig. 2d and Egger et al. 2006a,b), while decapitated animals were never observed to regenerate a head (Egger et al. 2006a,b). In this species, it seems that stimuli from the brain are required to duplicate or regenerate the brain. Is there a specific subset of predetermined “brain” neoblasts in species that cannot regenerate a brain? These neoblasts could be located in the vicinity of the brain and therefore lost by amputation of the brain. Brain excision experiments in polyclads, where only the encapsulated brain was removed (von Levetzow 1939; Koopowitz et al. 1976), do not support the existence of such brain-specific neoblasts, as these animals were not able to regenerate the brain. Also, amputation experiments in *M. lignano*, where only brain and the neoblast-free rostrum were amputated, showed no brain regeneration (Egger et al. 2006a,b). While neoblasts are required for regeneration in general, they do not determine whether a species is able to regenerate a specific organ such as the head, as was found with grafting experiments in triclads (Brøndsted 1969).

Can a single neoblast from an adult develop to a whole organism in an adequate environment, just like a zygote? For the parasitic flatworm *Taenia crassiceps*, it was shown that single cells of trypsinated cysticerci are able to produce complete cysticerci in the mouse host (Toledo et al. 1997). So in these cestodes, a single totipotent cell, other than a zygote, is able to recover a whole animal. No such experiment is known for free-living flatworms. Besides the difficulty in providing a suitable culture medium, distinct differences between a single neoblast and a zygote are yolk content or surrounding yolk cells and maternal determinants that are present in eggs, but not in neoblasts. Do (all or some) neoblasts have embryonic characteristics, as some authors held (e.g., Keller 1894)? When and where do neoblasts origin in the embryo? Polyclads with their clear and traceable embryonic development appear to be suitable flatworms for investigating these questions.

## Heteromorphoses

Heteromorphoses, i.e., the occurrence of supernumerary heads or tails, in species that cannot regenerate an amputated head, have shown that the regeneration potential of neoblasts is not

to be equated with the regeneration capacity of the species. Heteromorphoses are also a placative warning sign for the dangers coming with the ability to regenerate (all) organs. Last but not the least, heteromorphoses in free-living flatworms show that the duplication of axes is not only possible during embryonic development (Fig. 2b, siamese twins), but also in adults, even after comparably minor surgery. The *M. lignano* specimen depicted in Fig. 2c,d shows that a longitudinal incision in the anterior part of the animal can establish a second anteroposterior body axis parallel to the original axis. The new anteroposterior axis reaches from the rostrum tip to the caudal tip and duplicates all organs including head, pharynx, female genital opening, and male copulatory organ. Even the breadth of the animal approximates that of two single specimens. Over time, however, the animal degenerated in both halves losing all eyes, and was not able to recover to a viable state (Egger et al. 2006a,b). A decreased viability in animals with heteromorphoses was also observed in *Macrostomum appendiculatum* and *Bothrioplana bohemica* (Sekera 1911), as well as in *S. leucops* (Ruhl 1927b).

In triclads, duplicated or even multiple heads (e.g., 10-headed *D. lacteum*, Lus 1924) and tails have been generated and studied by a great number of researchers (see Brøndsted 1969). Randolph (1897) made longitudinal and lateral incisions in *Planaria maculata* [today: *Dugesia (Girardia) tigrina*] leading to additional eyes and pharynges. It was found that when a duplicated head or tail was amputated close enough to the second main axis, regeneration was inhibited by the presence of the remaining head or tail (Rand and Mildred 1926). In the polyclad *Leptoplana*, double-heads and double-tails were experimentally produced by longitudinal incision (Child 1905a). With the same method, tails, but not heads, could be duplicated in the rhabdoceol *Mesostoma* (Fulinski 1922) and in the acoel *P. caudatus* (Keil 1929). Contrastingly, heads, but not tails could be duplicated by incision in the catenulid *S. leucops* (Ruhl 1927b).

Another type of heteromorphoses is created by provoking a body axis reversed to the original body axis. This was found in *M. lignano* after longitudinal incision in the head, that induced the creation of a tail plate appendage in the anterior body region (Egger et al. 2006a,b). Agata et al. (2003) suggested that one of the main functions of a blastema in triclads is producing positional signals and inducing intercalary regeneration between stump and blastema. An incision may cause the building of a very small blastema at the wound site, which provides anterior or posterior positional signals. Depending on the signal the blastema produces, either a head or a tail plate is subsequently formed.

Incisions may also have a long-range effect, as was observed by Randolph (1897) in triclads, where outgrowths appeared at body regions not affected by incision. A similar phenomenon as in *M. lignano* was observed by Steinböck

(1967) in *H. gisela*, where an animal was divided into 10 pieces, one of which developed a tail-like structure at the anterior end besides the head. Just as was observed in *M. lignano* (Egger et al. 2006a,b), the anterior tail in *H. gisela* was gradually moving along the side of the animal towards the posterior end, when after 36 days the additional tail was located at the posterior tip of the animal.

## Conclusion

A pronounced regeneration capacity and the existence of possibly totipotent stem cells in adults are two key features of many flatworms not found in this combination in any other bilaterian taxa. It could be shown that regeneration follows similar principles in many different taxa of free-living flatworms, even if the regeneration capacity can vastly differ. Such comparisons make it possible to recognize unique patterns, and also to form general ideas how, why, and when regeneration works. For this reason, it appears to be valuable to establish several flatworm model organisms from various taxa, to address old and upcoming questions regarding regeneration and the neoblast stem cell system. One of these comparably new model flatworms is *M. lignano*, a small but versatile worm that facilitates regeneration studies on the cellular and molecular level.

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