

# The Role of Stem Cell Strategies in Coping with Environmental Stress and Choosing Between Alternative Reproductive Modes: Turbellaria Rely on a Single Cell Type to Maintain Individual Life and Propagate Species

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With 8 figures and 1 table

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**Abstract.** To contribute to a better understanding of the interrelationships of different forms of asexual and sexual reproduction in the Turbellaria, three selected species were studied with respect to the cellular correlates underlying the diverse reproductive strategies. The exclusively asexual freshwater triclad *Dugesia tahitiensis* holds the top position in regenerative and fissioning capacities within the Tricladida and, in addition, has the most neoblasts (stem or regenerative cells) known within this taxon. A reproductive rate of roughly one new specimen originating from a parent animal every third day at optimal experimental conditions correlated well with the speed of head regeneration ranging from 43 to 59 h at 23 °C. The process of fission (architomy) was observed and its dependence on population density and melatonin content was studied. In two new marine representatives of the Macrostromorpha from the Adriatic Sea, the distribution of neoblasts in the body and their fate during regeneration were traced by a recently developed labelling method with 5-bromo-2'-deoxyuridine. In *Macrostomum*, a sexual species with poor regenerative potential, the proliferating cells were localized predominantly along the nerve cords and migrated to their final location after mitosis. *Microstomum* n. sp., with asexual reproduction, showed a less concentrated pattern of proliferating stem cells, being localized in the future areas of organ primordia forming before fission in the process of paratomy. The findings are discussed with respect to cell biological, ecological and evolutionary implications.

## Problem

The mode of reproduction is intimately associated with environmental pressure. Sexual and asexual propagation have long been recognized as the two main alternatives. Keeping the propagative units as small as two single germ cells forming a zygote allows the production of a great number of offspring from two individuals; this maximizes the relation of both the possible number and the possible biomass between daughter and parent generation. The case is even more extreme when a population reproduces parthenogenetically, relying on just one cell for creating a new individual. In contrast, the diverse forms of asexual reproduction in a strict sense (*i. e.* not involving germ cells) have, in all multicellular organisms, in common that the propagative units consist of several, mostly of many cells. In quantitative respect, parthenogenetic reproduction seems to beat, and therefore should have outgrown, asexual forms in the course of evolution (Maynard Smith, 1979, 1989). In spite of this, diverse types of asexual reproduction are widespread in lower metazoans.

Short-term advantages of asexual reproduction and parthenogenesis contrast with long-term benefits of bisexual reproduction (Cockburn, 1991).

From a genetic point of view, the main discrimination separates (1.) sexual reproduction from two gametes forming a zygote and the types of parthenogenesis and pseudogamy with meiosis from (2.) asexual forms in a strict sense and all other forms using germ cells, but lacking meiosis and zygote formation (see Benazzi & Benazzi Lentati, 1993, for turbellarians). Meiosis and recombination preceding gamete formation and the combination of different maternal and paternal chromosomes in the zygote form the basis for a quantum step in genetic variability and, hence, plasticity and adaptive potential, when proceeding from asexual to sexual types of reproduction. This situation has fostered the opinion that sexual reproduction, being far more effective in creating new genotypes, is the original mode in extant metazoans and that reproduction without recombination (asexual or parthenogenetic forms) may at best exist for limited periods in the evolution of a species (Crow & Kimura, 1965). To understand its coexistence with sexual reproduction over rather long evolutionary periods, the advantage of asexual reproduction has been correlated with the requirements of special environmental situations (*e. g.*, Calow *et al.*, 1979).

Less effort has been invested in elucidating the cell biological and genetic basis and correlates of asexual forms of propagation. Therefore, it appeared attractive to contribute to this issue by studying a taxonomic group with high regenerative potential relying on stem cells. In addition to sexual reproduction, the chosen group includes a variety of asexual forms, often in closely related or even in the same species. The following favorable prerequisites supported choosing turbellarians for such a study: the discovery of a purely asexual planarian species that may easily be reared in laboratory cultures (Gourbault, 1977), the existence of considerable knowledge on life cycles and cytogenetics of triclads (Benazzi & Benazzi Lentati, 1976), the availability of laboratory cultures of Macrostomorpha (Rieger *et al.*, 1988) and the occurrence of both sexual and asexual reproduction in closely related macrostomorph species whose strategies in cell renewal and reproduction are well documented (Palmberg, 1986; Palmberg & Reuter, 1983). The comparison of the only triclad species known to reproduce exclusively asexually with other planarian species using both sexual and asexual reproduction modes should provide a better insight into the biological relevance of these differing strategies,

including the ecological implications. Among the Macrostromorpha, with their phylogenetic position at the base of the Rhabditophora, the marine representatives investigated in this study offer the advantage that an established protocol exists to visualize DNA synthesis; in connection with the small size of these animals, this permits to follow cell fates in whole mount preparations (Ladurner *et al.*, 2000).

If one trusts the currently prevailing opinion on metazoan phylogeny, then the Platyhelminthes also offer the advantage of originating near the common root of all bilaterians (Carranza *et al.*, 1997; Ehlers, 1985; Rieger, 1985; Ruiz-Trillo *et al.*, 1999); knowledge derived from this taxon might therefore enhance our understanding of similar issues in other animal phyla. The regular occurrence of both sexual and asexual reproduction in the life cycles of parasitic forms like trematodes and cestodes (Ehlers, 1985) may offer an additional help in theoretical deductions (see Discussion). Within the platyhelminths, the cellular basis of both reproductive modes has been studied best in the paraphyletic group of the Turbellaria. A cell type termed neoblast (Baguña, 1981; Brøndsted, 1969; Peter, 2001), which is claimed to be totipotent, gives rise to both somatic cells and to gametes. This implies even a cellular link between alternative forms of reproduction.

## Material and Methods

The freshwater planarian *Dugesia tahitiensis* Gourbault 1977 (Tricladida Paludicola) was obtained from the collection undertaken for the description of this endemic species (Gourbault, 1977). Experimental animals (Fig. 1) measuring from 9.5 – 12.5 mm in length were selected from a laboratory stock kept for many years in the dark at 19 °C in Mc Connell's artificial pond water (Mc Connell, 1967) and were fed once a week with tubificid worms obtained commercially. To minimize variation of nutritive effects within the single experimental series, the same food supply was used for each series. Only planarians with a pointed tail were taken and those with a blunt end rejected. This excluded animals that had fissioned recently and not yet regenerated completely. In the fission experiments, the planarians were fed in intervals of 4 days and exposed to a temperature of 23 °C ± 0.5 °C and a photoperiod of 12 h per day (daylight fluorescent bulbs, 700 – 1,400 lx). Water was changed in the mid of each feeding interval. The experimental temperature coincided well with the range of 23–24 °C reported for the original habitat. Cohorts of different numbers were kept in glass Petri dishes of varying diameter, so that they differed in the area available per worm. The water volume was adjusted so that there were 10.0 ml of water per animal. This enabled the effect of population density (crowding) on fission to be tested. In intervals of 1 – 2 days, the posterior fission products were counted in each dish and separated. The influence of different concentrations of melatonin in the culture water was studied with the same experimental design and under the same conditions.



Fig. 1. *Dugesia tahitiensis*, dorsal view of live specimen; scale: 3 mm. From Peter & Schürmann (1995).

The behaviour preceding fission and the fission process were observed under ambient light (daylight fluorescent bulbs) in selected specimens showing a constriction postpharyngeally. For documentation, photographs were taken using a Zeiss Tessovar closeup lens equipped with a Contarex camera and a ring flash.

The sexual *Macrostomum* n. sp. (a representative of the *M. tuba* clade) and the asexual *Microstomum* n. sp. originated from a collection in the Adriatic Sea near Lignano, Italy. *Macrostomum* was reared as described previously (Rieger *et al.*, 1988). The experimental animals were kept at 20 °C, fed with diatom cultures and starved 2 days before labelling.

In a pilot study, data were obtained to correlate cell biological parameters to reproductive peculiarities. For this purpose, neoblasts were isolated by mechanical disintegration in a Dounce homogenizer with a loosely fitting pestle (overall tolerance of 50 – 70 µm) and studied as described elsewhere (Schürmann *et al.*, 1998; Behensky *et al.*, 2001; Peter, 2001). For *Dugesia tahitiensis*, the neoblast percentage was compared to values published for comparable species. In the macrostomid species, proliferating cells were labelled with 5-bromo-2'-deoxyuridine and their localization in different body regions was traced by an appropriate immunocytochemical technique (Ladurner *et al.*, 2000). Analytical grade reagents were obtained from Sigma; where available, cell culture tested compounds were chosen.

## Results

### 1. Fission frequency in *Dugesia tahitiensis*

Animals kept singly in 10 ml of water in Petri dishes with an area of 44.2 cm<sup>2</sup> fissioned, on the average, every third day (fission rate: 0.32 ± 0.03 per day per specimen). A slight crowding effect was observed when 30 animals were held in 6.8 cm<sup>2</sup> (Fig. 2). This dif-

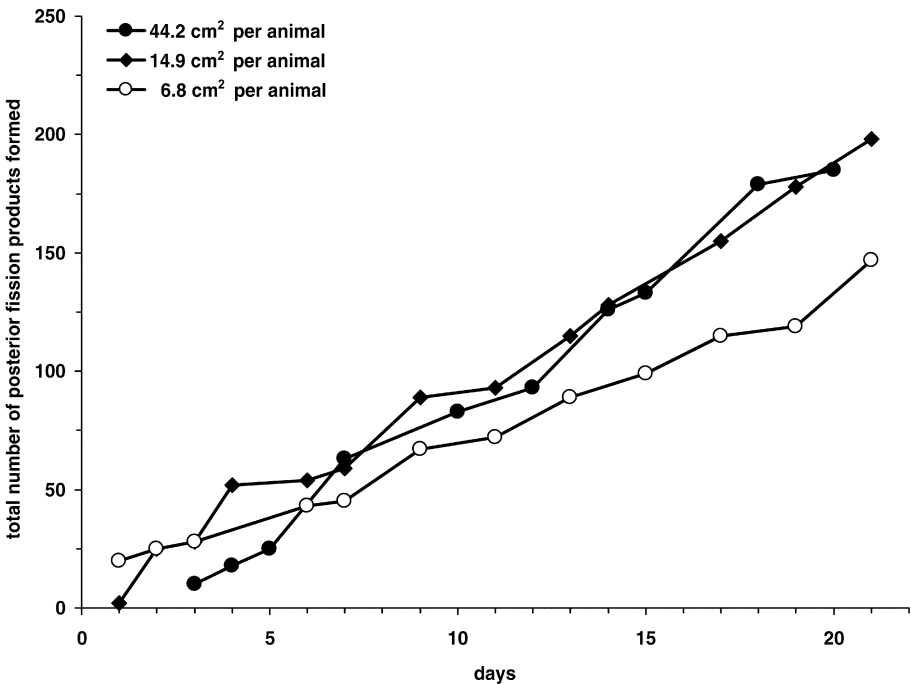


Fig. 2. Population growth in three different groups of *Dugesia tahitiensis* kept at different population densities. 44.2 cm<sup>2</sup> per animal; 1 planarian per Petri dish, a total of 29 dishes. 14.9 cm<sup>2</sup> per animal; 6 planarians in each of 5 dishes. 6.8 cm<sup>2</sup> per animal; 30 animals in a glass cylinder.

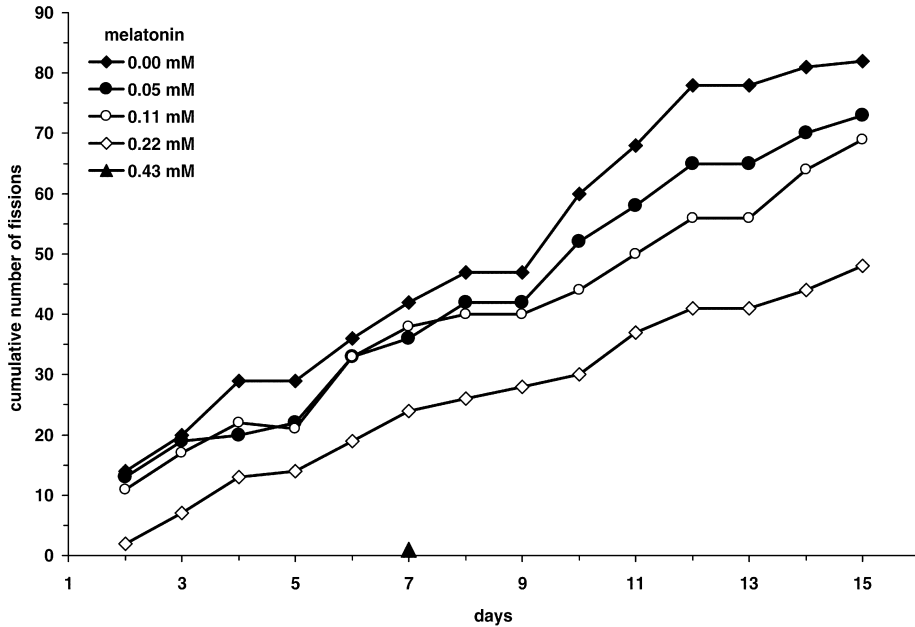


Fig. 3. Population growth in cohorts of *Dugesia tahitiensis* at four different concentrations of melatonin dissolved in the culture water. To counteract microbial degradation, 0.02% (w/v) of neomycin sulfate was added. 3 groups of 7 planarians per dish (6.9 cm<sup>2</sup> per animal) were used for each concentration. All fission numbers relate to the total of 21 planarians per individual concentration. The single triangle near the baseline marks the only fission that occurred at a concentration of 0.43 mM.

ference was, however, statistically not significant. A direct effect of group size *per se*, in addition to the available area, was ruled out. In another series of experiments, a further, statistically significant decrease in fission rate to 0.17 per day per animal had been recorded when the available area was reduced to 2.5 cm<sup>2</sup> or 1.3 cm<sup>2</sup> per specimen (Peter, 1994). This reduction to 53% of the maximal reproduction rate is a rather modest crowding effect in comparison with other species (see Discussion). Different supplies of tubificid worms yielded different fission rates.

Melatonin inhibited fission nearly completely at a concentration of 0.43 mM (100 ppm) in the culture water. This inhibition was highly significant compared with the controls without melatonin ( $P = 0.01$ ; t-test). A slightly significant reduction in fission rate ( $P = 0.1$ ; t-test) was observed at a concentration of 0.22 mM (Fig. 3).

## 2. The fission process in *Dugesia tahitiensis*

The observations fit into the scheme of architomy (Brøndsted, 1969). Before fission, the animals showed a constriction in the postpharyngeal region. The “waists” varied in position and form, from slight narrowings and finlike tails (Fig. 4A) to a very thin isthmus (Fig. 4C). Such animals often performed twisting movements and adhered firmly to the bottom of the dish with their tail region, while the anterior end crept forward (Fig. 4B). At other times, the anterior end was creeping, while the tail lost contact to the

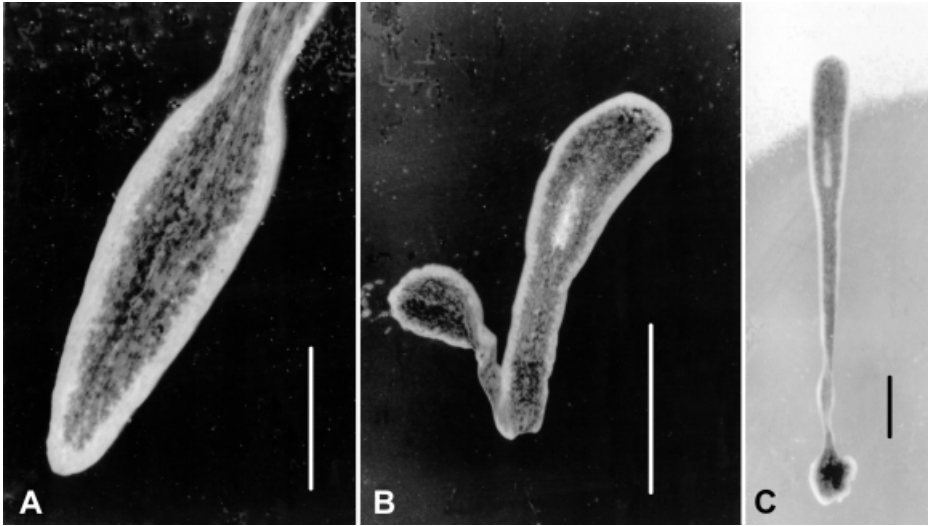


Fig. 4. Selected stages in the fission process of *Dugesia tahitiensis*. A: Finlike tail (scale: 1 mm); B: Specimen in twisting movements (scale: 3 mm); C: Animal with the tail adhering firmly to the bottom of the culture dish, while the rest of the body is stretching extremely (scale: 5 mm).

bottom and was held upwards. Altogether, the impression of a lack in coordination between anterior and posterior body regions prevailed and confirmed observations from other planarian species (see Discussion). The body was frequently stretched to considerable lengths (Fig. 4C). It was, however, not possible to predict the moment of fission from the appearance of this isthmus: even extremely thin constrictions often persisted for hours. Therefore, quantitative data on the duration of the entire fission process could not be derived from the visual observations. In one case, fission itself was observed and documented (Peter, 2001; Peter & Schürmann, 1995). It consisted in a mechanical tearing apart of the animal at a narrow isthmus within a split second: While the tail stayed in place, the anterior fragment crept away. The posterior fragment moved on, too, though somewhat later and slower.

Fission always occurred postpharyngeally. The distance of the fission plane from the tail tip varied from about one third to one fifth of total body length. Posterior fragments developed a blastema and regenerated a head within about 2 days in a process similar to regeneration after transversal cutting (Fig. 5; cf. Peter, 1995, 2001). Frequently, posterior fragments divided transversally before regeneration of a head. In these cases, the middle fragment was discernible by two blunt ends (Fig. 6).

### 3. Neoblasts in *Dugesia tahitiensis* and in Macrostromorpha

Apart from the size difference, the morphological appearance and specific staining were identical for neoblasts from *Dugesia tahitiensis* (diameter: 8–12  $\mu\text{m}$ ) and *Macrostromum* (diameter: 3–4  $\mu\text{m}$ ). The cytological findings fitted into the typical neoblast image (Fig. 7). In *D. tahitiensis*, 44 % of the total cell count were neoblasts (Table 1). In the sexual *Macrostromum*, synthesis of DNA traced by the incorporation of 5-bromo-2'-

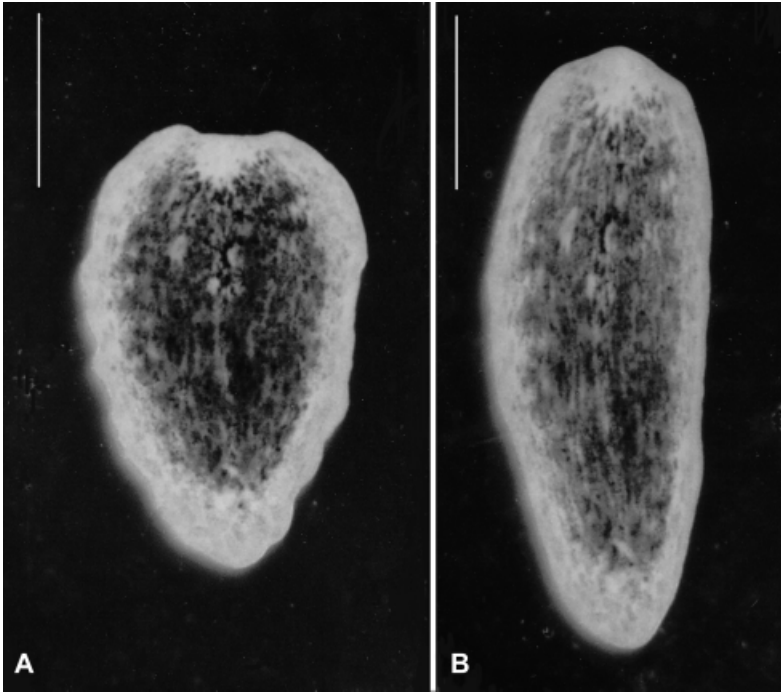


Fig. 5. Regenerating posterior fission products of *Dugesia tahitiensis*. A: 1 h, B: 53 h after the fission. Tiny eye spots are visible in the regenerated head in B (scale: 1 mm).

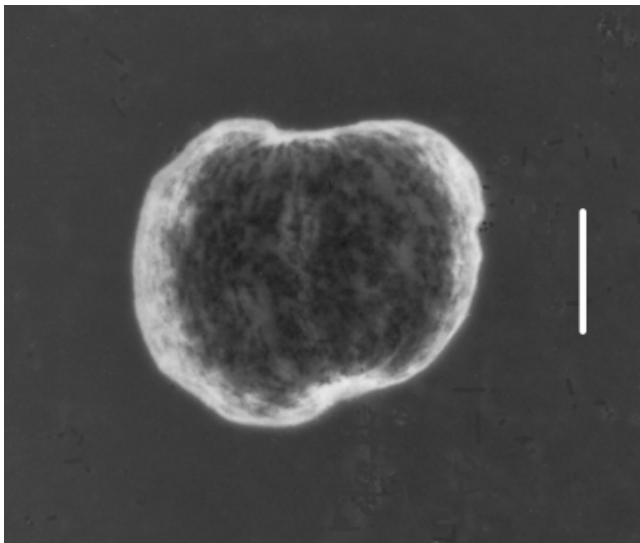


Fig. 6. Anterior fragment resulting from the fission of a posterior fission product of *Dugesia tahitiensis* before a head had been regenerated. Front end upside. Note that both ends are blunt (scale: 1 mm).

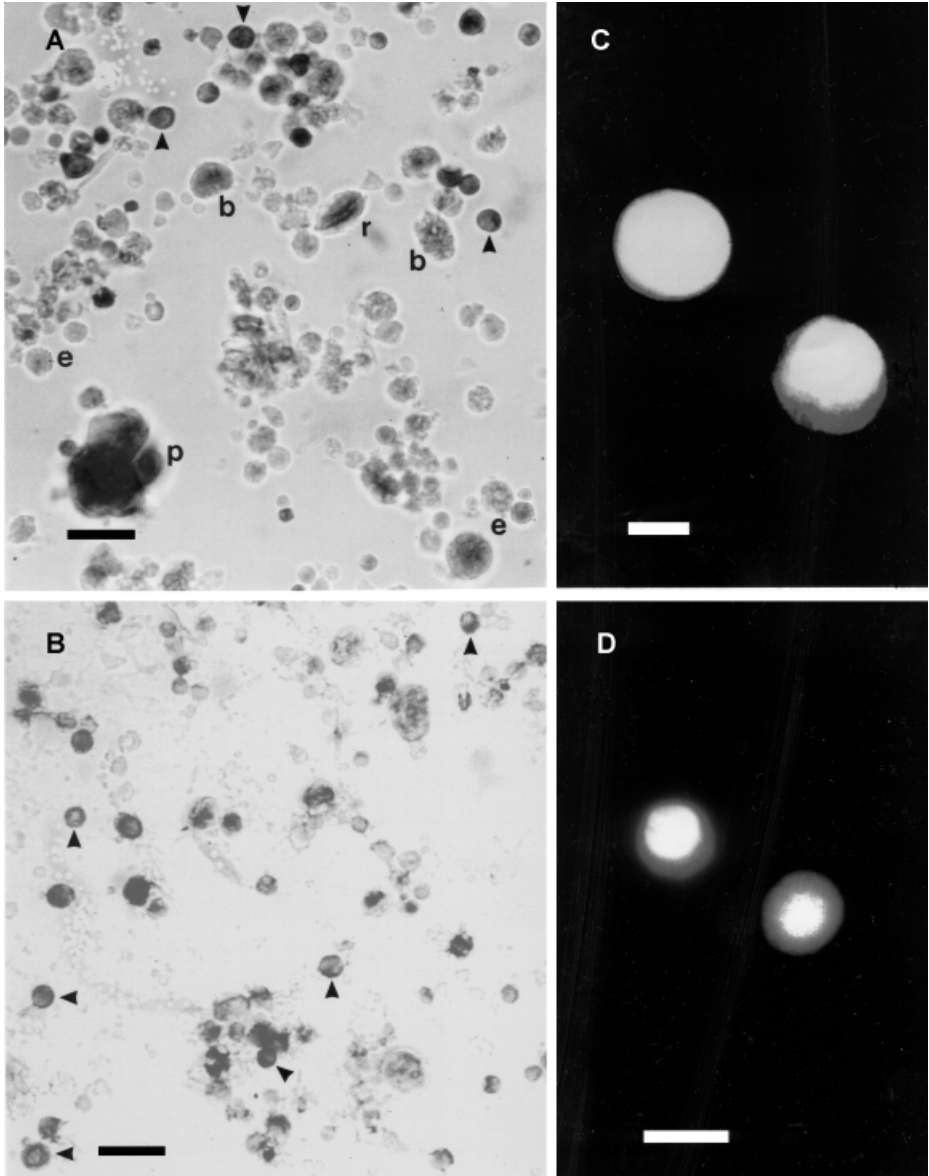


Fig. 7. Cell suspensions prepared from selected Turbellaria. A, B: Cells from *Dugesia tahitiensis* isolated as described in the text and stained differentially for neoblasts with azure A – eosin B (Pedersen, 1959); scale: 30  $\mu$ m. A: Partially purified suspension from filtration through a 40  $\mu$ m mesh net; B: Preparation enriched in neoblasts after further filtration through a net (gauge: 15  $\mu$ m). Neoblasts recognizable in both preparations by their narrow and strongly basophilic cytoplasmic rim appearing dark in the photograph (arrowheads). Further cell types may be identified in A: basophilic gland cells (b), epithelial cells (e), a parenchymal cell (p) and rhabdoid forming cell (r). The applied dye mixture is, however, optimal only for neoblasts. C, D: Selected neoblasts purified by gradient centrifugation and stained with the fluorescent dye acridine orange. Documentation by video microscopy (Behensky *et al.*, 2001), showing different nucleocytoplasmic ratios in *Dugesia tahitiensis* (C) and *Macrostomum* n. sp. (D). The red cytoplasm shows up as a dark rim against the bright nucleus with its green fluorescence adding to the red of the overlying cytoplasm. Scale: 5  $\mu$ m.

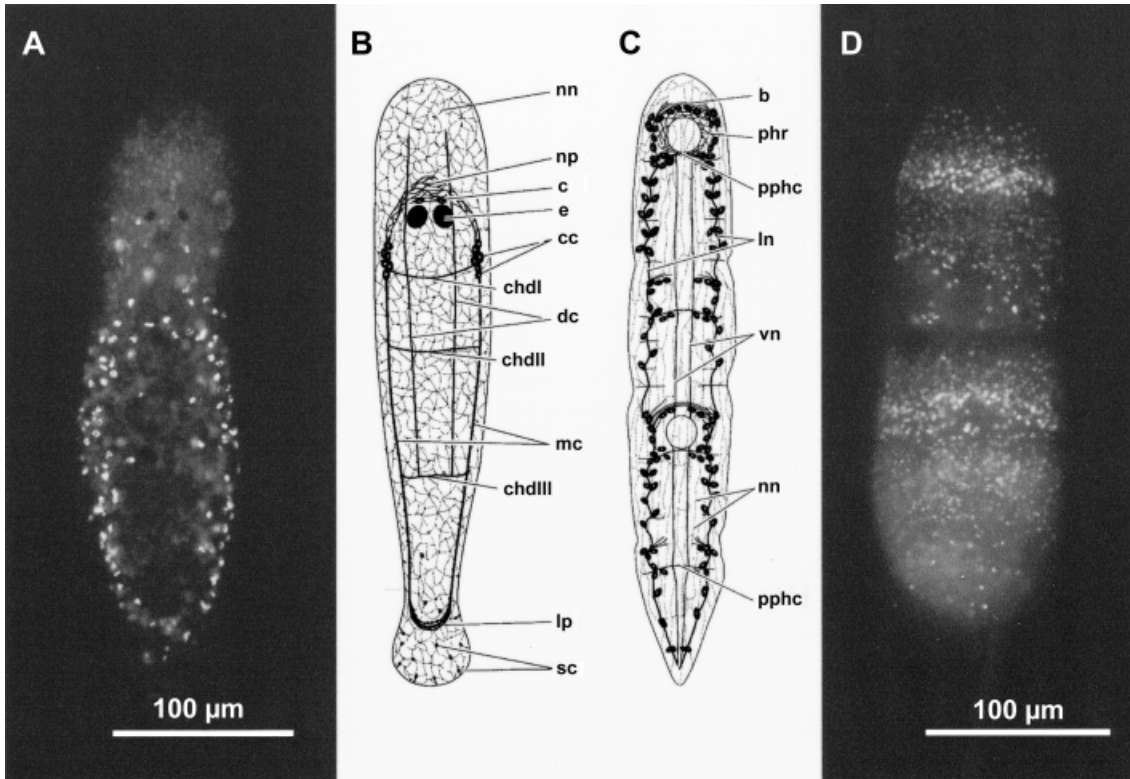


Fig. 8. Distribution of proliferating neoblasts (A, D) and organization of the nervous system (B, C) in two genera of the Macrostomomorpha: *Macrostomum* (A, B) and *Microstomum* (C, D). Proliferating cells (neoblasts) have incorporated 5-bromo-2'-deoxyuridine and may be recognized with a confocal microscope by their bright fluorescence against the dark background after immunocytochemical staining (Ladurner *et al.*, 2000). Their distribution in the body differs between the two marine species *Macrostomum* n. sp. (A) and *Microstomum* n. sp. (D). Whereas proliferating cells in the purely sexual *Macrostomum* n. sp. (A) are concentrated along the main nerve cords (mc) visible in the scheme for *Macrostomum hystricinum marinum* Rieger 1977 (B), labelled cells show a different pattern for *Microstomum* n. sp. (D). In the latter genus, whose nervous system (scheme for *Microstomum lineare* (Müller 1774) in C) differs from *Macrostomum*, the pattern lacks an apparent correlation to the nerve cords. Rather, it resembles the pattern of future division planes between the forming zooids in *Microstomum lineare* that are visible as lateral indentations in the scheme (C). Reproduction in the new marine *Microstomum* has not yet been studied. The freshwater species *Microstomum lineare* may reproduce asexually by paratomy (transversal fission), after the anterior ends of new zooids have been formed by organ primordia originating from neoblasts (Palmberg & Reuter, 1983). b, brain; c, cells above eyes; cc, cluster cells; chdI, chdII, chdIII, first, second and third dorsal commissure; dc, dorsal cords; e, eyes; ln, lateral nerve cord; lp, loop; mc, main cords; nn, nerve net; np, neuropile; phr, pharyngeal nerve ring; pphc, postpharyngeal commissure; sc, sensory cells; vn, ventral nerve cords. B. and C. from Ladurner *et al.* (1997).

deoxyuridine was found predominantly along the lateral longitudinal nerve cords. The head region was completely free of these cells. Such cells migrated into other body regions, and the label was ultimately found in differentiated cells such as epithelial cells or newly regenerated tail structures (Ladurner *et al.*, 2000). In contrast, specimens of *Microstomum* that lacked gonads showed a less restricted distribution, with labelled cells incorporating label also in median body regions (Fig. 8).

## Discussion

### 1. Asexual versus sexual reproduction and the reproductive potential of architomy in the Turbellaria

As argued above, a naive view might postulate that sexual reproduction should have displaced asexual forms in multicellular organisms over the course of evolution. What, then, may explain the existence of asexual reproduction in many metazoan taxa? Some arguments are evident: Stages in asexual propagation surpass comparable zygotes in mass, potentially reducing the danger of death by environmental stress, especially by predation. As asexuality and, in part, also parthenogenesis and pseudogamy imply the formation of clones, maintaining an optimized genotype with advantageous heterozygous gene combinations may be one important function of these reproductive strategies (Cockburn, 1991). Both arguments explain preconditions for survival in “extreme” environments (“extreme” being defined with respect to the original adaptations of the species). This view is strengthened by the frequent observation that laboratory cultures of sexual planarian strains change to asexual reproduction.

A consideration correlating bioenergetic facts to ecological conditions has led to the conclusion that “egg production is more efficient in converting food to reproductive energy but fission is more efficient at converting this to offspring” (Calow *et al.*, 1979, p. 715). Therefore, fission should be favored as trophic conditions deteriorate and be more frequent in stream-dwelling triclads than in inhabitants of lakes. Such a correlation does, in fact, exist and applies also to *Dugesia tahitiensis*, which is found in streams. Whereas gamete production apparently costs less energy than differentiation of the somatic tissues involved in growth and hence also in fission, the development of embryos requires additional energy; this ultimately makes sexual reproduction more expensive. The greater fitness of larger offspring in contrast to small hatchlings (Smith & Fretwell, 1974) may add a further advantage. With nutrient restrictions, note that relative metabolic intensity is higher for small triclads (Bertalanffy, 1942), favoring larger offspring in poor environments.

It might be argued that, under appropriate nutritional conditions, egg production should yield more offspring than fission because hatchlings are smaller than fission products. Too few data on planarian generation times and growth rates exist to draw firm conclusions. The very high frequency of fissions observed in *D. tahitiensis* under the experimental conditions (Fig. 2), with a maximum of about one posterior fragment generated every third day, suggests that this species might approach the reproductive potential of comparable sexual species. This would explain the complete lack of sexual reproduction and the failure of treatments that sexualize other fissiparous planarian strains (Grasso & Benazzi, 1973). For the predominantly asexual *Girardia (Dugesia) dorotocephala* (Woodworth 1897), the maximal fission rate is roughly 0.2 per day per animal (Best *et al.*, 1974). This corresponds to a reproduction rate of about 18 posterior fission products per parent animal in 13 weeks. In extensive experiments with a sexual population of the same species, an average of 19 hatchlings were counted for one planarian in 13 weeks. This number has been calculated from reproductive rates during the first three years in the life of the animals (Jenkins, 1974, p. 504–505). An analogous calculation with the fission rate found for *D. tahitiensis* gives 29 fissions per planarian in 13 weeks. Clearly, definitive conclusions should not be drawn from this estimation,

Table 1. Pilot count of cell types in *Dugesia tahitiensis*, compared with the respective percentages reported from two species of Dugesiidae. To identify the cells, a microscope equipped with phase contrast optics was used. Maceration and fixation of tissues and comparative counts for *Schmidtea mediterranea* and *Girardia tigrina* were adopted from Baguña & Romero (1981).

cell type	% of total cell count in			
	<i>Dugesia tahitiensis</i>	<i>Schmidtea mediterranea</i>		<i>Girardia tigrina</i>
body length:	8 mm	7 mm	16 mm	7 mm
neoblast	44	28	18	32
nerve	6	16	8	22
epithelial	7	13	11	12
parenchymal	3	15	25	10
acidophilic gland	4	6	9	3
basophilic gland	5	4	8	3
pigment	1	1	2	1
muscular	9	3	2	2
gastrodermal	3	4	6	4
goblet	2	1	1	1
flame	1	1	2	–
rhabdite	3	2	2	10
striped	2	1	1	–
unclassified	10	5	5	–

as experimental conditions are not strictly comparable and information on the crucial parameter generation time is lacking. In addition, fission frequency and growth rate of the posterior fragments are not known, and the step from laboratory experiments to natural conditions is big. Nonetheless, *Dugesia tahitiensis* has the most rapid asexual reproduction among the triclads. Its reproductive potential is also extraordinary in another respect: even at high population densities (one animal per 1.3 cm<sup>2</sup>), fission was inhibited to less than 50%. In contrast, *Girardia (Dugesia) dorotocephala* stopped fissioning completely when a dish contained one animal per 4.1 cm<sup>2</sup> (Best *et al.*, 1974).

Whereas architomy with regeneration of the missing body parts following fission appears to be an optimal strategy in stream habitats, paratomy seems less well adapted to such habitats. This form of fission, with the development of new organs before fission, is plausibly favored in the more sheltered interstitial environments encountered by psammobionts. A reduced fitness of the intermediate, possibly less mobile zooid stage might be less critical for these organisms (Calow *et al.*, 1979).

## 2. Physiology of the fission process in triclads

The influence of melatonin and the fission mechanism observed in *Dugesia tahitiensis* confirm results obtained with *Girardia (Dugesia) dorotocephala* (Morita & Best, 1984). Melatonin occurs in planarians and thus appears to be a phylogenetically old

regulatory molecule in reproductive biology (Morita & Best, 1993). Mechanical tearing was used to explain fission in planarians by Child (1910), and a process similar to that reported here, with a very rapid and unpredictable final separation of both fragments, was observed for an asexual strain of *Dugesia gonocephala* Dugès 1830 by Pala *et al.* (1987). In this strain, the posterior fragment remains immobile until a head is regenerated and fission cannot occur without a head. In contrast to this finding, the absence of a head seems to facilitate fission in *Dugesia tahitiensis*, as has also been described for *Girardia (Dugesia) dorocephala* (Best *et al.*, 1974). The occurrence of two consecutive fissions before the posterior fragment has regenerated a head is reminiscent of the acoelomorph *Convolutriloba longifissura* Balzer & Bartolomaeus 1997, where a longitudinal fission of the posterior derivative follows the transverse fission of the intact animal (Åkesson *et al.*, 2001). The tearing process in triclad s has been ascribed to a reduced neural coordination between the brain and posterior body regions (Best *et al.*, 1974). An ultrastructural correlate has been found recently in *Dugesia japonica* Ichikawa & Kawakatsu 1964, where gap junctions occur in the parenchyma in the future fission region (Hori & Kishida, 1998).

The inhibition of fission in dense populations may stem from close encounters between planarians – a social control of fissioning (Pigon *et al.*, 1974). It could help regulate population density in nature. The lack of relevant observations in the natural habitats currently precludes interpreting the different behavior shown by *D. tahitiensis*.

### 3. Stem cells, reproduction and regeneration

The neoblast was originally described as a cell being intimately related to regeneration. In histological sections, early stages of regeneration blastemas in planarians consist of relatively small cells with prominent nucleoli and a narrow, strongly basophilic cytoplasmic rim. Similar cells, spherical to pear shaped, are abundant in the parenchyma (for a review, see Brøndsted, 1969). Later ultrastructural investigations revealed a cell type with minimal cytoplasmic differentiation, free ribosomes and mitochondria being the only organelles (Hori, 1992, 1997). More recently, evidence for heterogeneity of the neoblast pool was found, and this cell type was characterized cytologically also in other turbellarian taxa (see, for example, Baguña *et al.*, 1988, 1994; Gschwentner *et al.*, 2001; Peter, 2001; Rieger *et al.*, 1999; Schürmann *et al.*, 1998).

In addition to the architypy that is typical for the Tricladida (see above), a second form of fission is known from turbellarians: Paratomy consists in the formation of organ primordia along the longitudinal axis, with transversal fission following when the new structures have been completed. This mode of reproduction, which can form zooids, is present in the Macrostomorpha, *Microstomum lineare* being a classical representative (see, for example, Palmberg, 1990). The involvement of neoblasts in paratomy has been traced in the freshwater turbellarian *Microstomum lineare* by autoradiography (Palmberg & Reuter, 1983); this strategy is likely for the marine *Microstomum* n. sp. based on the distribution of proliferating cells described in this paper. 5-bromo-2'-deoxyuridine offers the advantage of a higher resolution than <sup>3</sup>H-thymidin.

Regeneration in planarians has decisively impacted the development of modern regeneration science, beginning with the extensive studies by Thomas Hunt Morgan and Charles Mannor Child (see Brøndsted, 1969, for a review and further references). Pla-

narians present a mixed regeneration type, combining epimorphosis with morphallaxis: The regeneration blastema, the source for the new tissues, is formed *de novo* from neoblasts after preceding mitoses (Baguña, 1998). The subsequent remodeling that eventually restitutes the former body proportions (Mead, 1985) is based on already existing tissues. High regenerative potential not only enables rapid repair of artificial traumata, but is correlated with the possibility to reproduce asexually and, in the extreme case of *Dugesia tahitiensis*, even with obligatory asexual reproduction. This facilitates recognizing a biological function exerted by extraordinary regenerative capabilities. *Dugesia tahitiensis* possesses maximal regenerative capacity among the Tricladida Paludicola and regenerates in a similar way after injuries and after fission. Tiny new eye spots are formed within 43–59 h, and pieces cut at any level along the longitudinal body axis, except the head and the utmost tail tip, regenerate completely (Peter, 1995, 2001).

The current view of the neoblast and its function within the diversity of turbellarian cells may be summarized briefly as follows: The turbellarian body consists of a functional and a proliferative compartment (Baguña, 1998). Differentiated cells build up the former, whereas – with the exception of germ cells – the only cells capable of mitosis are the neoblasts (Baguña *et al.*, 1994). These constitute a heterogeneous population comprising true stem cells, progenitor cells for diverse tissues and differentiating cells (discernible ultrastructurally by the occurrence of endoplasmic reticulum and Golgi structures: Hori, 1997). All differentiated cells originate from neoblasts, the ultimate origin being the true stem cells among them (Baguña *et al.*, 1988, 1994). Should there be a single stem cell line, it would be totipotent in a strict sense: it would have the potential to form all other cell types. Otherwise, several morphologically similar stem cell types would form a totipotent proliferative compartment. Our current research activities strive to solve this issue (Peter, 2001). That neoblasts can form gametes is proven by a series of observations and experiments, among them the absence of a separate, embryologically traceable germ line (Ehlers, 1985), the complete reduction of gonads when sexual planarians are starving and their reconstruction upon feeding (Stoppenbrink, 1905) and the sexualization of asexual strains by feeding them with sexual specimens from other species (Grasso & Benazzi, 1973). The role neoblasts play in continuous cell renewal (“physiological regeneration”) and in regeneration after wounding is well established (for reviews, see Baguña, 1998; Brøndsted, 1969). Considerable controversy exists, however, on their origin (for reviews, see Baguña *et al.*, 1988, 1994; Gremigni, 1981, 1988; Peter, 2001); most authors favor the existence of a permanent, self-renewing pool of totipotent stem cells (see, for example, Baguña *et al.*, 1989). Whatever their real origin, the stem cells among the neoblasts are the cellular basis for both sexual and asexual reproduction in turbellarians, at least in those cases where appropriate cell biological analyses have been performed. Note that major taxa, *e. g.* the Acoelomorpha, remain poorly studied in this respect. Incorporation of 5-bromo-2'-deoxyuridin into proliferating cells promises new insights into the cell kinetics of this taxon (Gschwentner *et al.*, 2001). Differentiated somatic cells are clearly incapable of dividing in turbellarians. This points to a general stem cell strategy that is competent for renewal during somatic cell turnover, for wound repair and posttraumatic restitution of body parts and for all methods of reproduction. This helps explain the ease of the switch between sexual and asexual reproduction in turbellarians.

Thus, the results described in this paper can best be explained by a high density or/and a special distribution of neoblasts as prerequisites for asexual reproduction by fission (architomy or paratomy). The results also support the key role of neoblasts in asex-

ual reproduction. The cell counts of *Dugesia tahitiensis*, when compared to previous reports for other planarians (Baguña & Romero, 1981), corroborate the importance of a high neoblast density in the parenchyma as a prerequisite for efficient regeneration after fission. Note again that the only species in the Tricladida Paludicola with exclusively asexual reproduction has the highest fission frequency, regeneration potential and neoblast content. The latter factor is certainly not the only one responsible for the asexual state. Other purely sexual triclads (e. g., *Schmidtea [Dugesia] polychroa* [Schmidt 1869]) exhibit regenerative capacities and neoblast densities comparable to those in species with both sexual and asexual reproduction (Brøndsted, 1969; Romero & Baguña, 1991). Additional genetic factors must be responsible for the option or switch between the two reproductive modes (Benazzi & Benazzi Lentati, 1993). The action of both environmental and genetic factors has been demonstrated in experiments on the sexualization of asexual planarian strains (Grasso & Benazzi, 1973). Different strains react differently to various treatments, and *Dugesia tahitiensis* resists a sexualization treatment completely (Nascetti *et al.*, 1990).

#### 4. Evolutionary implications

The evolutionary implications – given the dead end which asexual reproduction represents with respect to recombination – are of special interest in the present context. Whereas the existence of sympatric or allopatric sexual populations of other triclads guarantees their persistence, *Dugesia tahitiensis* apparently lacks such a possibility. This endemic population was probably introduced to Tahiti by the Polynesian immigrants, and its isolation is thus rather recent. This follows from the absence of close marine relatives, the relatively recent volcanic formation of this oceanic island far from any continent and the lack of other than human dispersion mechanisms over long distances (Gourbault, 1977). Nonetheless, no closely related species has yet been identified, although the *Girardia (Dugesia) dorotocephala* group shows some similarities with respect to isoenzyme polymorphisms (Nascetti *et al.*, 1990). One potential mechanism that might somewhat compensate for the lack of recombination, gamete and zygote selection in asexual reproduction – but whose existence remains to be confirmed – is a permanent selection of stem cells with reduced viability. This would avoid the accumulation of genetic lesions over the course of subsequent cell divisions.

Little information is available on the interrelationships between neoblasts and paratomy beyond the fact that a sufficient supply of stem cells is a basal requirement for the organ primordia. The present study does point to a correlation between paratomy and the spatial pattern of proliferating cells within a turbellarian: The concentration of proliferative cells along the longitudinal nerve cords in *Macrostomum* (purely sexual) contrasts well with the less concentrated distribution in *Microstomum* (sexual/asexual) (Fig. 8). Whereas neoblasts must migrate in order to reach the regeneration blastema in the former (Ladurner *et al.*, 2000), the primordia needed for asexual reproduction in the latter may form from neoblasts already in position.

Can conclusions on the reproductive mode in the bilaterian stem species be drawn from the turbellarian data? Hypothetically, the view that the stem species relied on three rather than two cell types for propagation (Rieger, 1986; Rieger & Weyrer, 1998) and thus had the option of asexual versus sexual reproduction is intriguing. Two gametes

and an “asexual spore”, the neoblast (precisely the stem cell[s] among the neoblasts), are used for propagation alternatively in many turbellarian species. Populations may thus take full advantage of either reproductive mode, depending on physiological or ecological necessities (Cockburn, 1991). This view is corroborated by the obligatory occurrence of both modes in the life cycles of parasitic Neodermata within the Platyhelminthes and by the identification of neoblasts as the only source of differentiated cells in the Cestoda (Wikgren & Knuts, 1970, among others).

## Conclusions

Close correlations between totipotent stem cells (neoblasts), regeneration and reproductive mode were found in three representatives of the Macrostomorpha and Tricladida. Reproductive strategies, in turn, are known to be related to environmental conditions. In turbellarians, neoblasts give rise to both somatic tissues and gametes. *Dugesia tahitiensis* reproduces only by fission (architomy) and occupies top positions within the Tricladida when considering neoblast count, regeneration potential and speed of reproduction. This underlines the common key role neoblasts play both in regeneration (reparative and physiological) and reproduction. The results show that a higher percentage of neoblasts promotes regeneration and reproduction, and are compatible with the fact that additional endogenous and exogenous factors regulate the reproductive mode. In Macrostomorpha, the distribution of proliferating neoblasts in *Microstomum* n. sp. indicates the prospective division pattern in paratomy (organ primordia formed before fission). In the exclusively sexual *Macrostomum* n. sp., proliferating cells are concentrated along the longitudinal nerve cords. Overall, a stem cell strategy appears to integrate vital somatic and generative functions and, in this way, both asexual and sexual reproduction in turbellarians. Adapting reproductive strategies to environmental needs therefore inevitably implies the involvement of stem cells. As recombination is probably indispensable in the long run, the evolutionary fate of exclusively asexual species, like the endemic *Dugesia tahitiensis*, cannot be predicted at present.

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