Sexual Differentiation and Germ Cell Transfer in the Colonial Ascidian *Botryllus schlosseri*

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ABSTRACT   The sexual differentiation in the colonial ascidian *Botryllus schlosseri* was considered with respect to colony sexualization and sexualization of the single zooids of the same blastogenic generation.

The colony sexualization was investigated histologically in a series of young colonies reared in the laboratory and fixed periodically throughout a sequence of blastogenic generations. The newly set colonies lack gonad blastemata; next, germ cells appear and several generations are involved in various phases of hyposexuality prior to the appearance of generations in which mature both eggs and sperm.

The sexual contribution of the zooids of the same generation was found to vary according to an intrinsic bilateral asymmetry (the gonadogenic potential differs both contralaterally in each zooid and between zooids of a different, dextral or sinistral, parental origin) and in proportion to the quantity of germ cells that have been transferred from preceding generations.

The transfer of germ cells from one to the following generations was investigated by fusing colonies of opposite pigment genotypes, then crossing the separated parabionts to a common genotype, and scoring the offspring pigment phenotypes. This experiment has shown that not only immature oocytes but also undifferentiated germ cells were exchanged between the parabionts and then gradually matured as eggs or sperm in a prolonged series of generations.

The primordial germ cells differ in size from hemoblasts, the source of hemocytes. However, further experiments are needed in order to decide between the two contrasting hypotheses of germ cell origin: either segregation from the atrial epithelium of buds or development from hemoblasts.

The sexual differentiation in the colonies of *Botryllus* has been recently re-considered by Mukai and Watanabe ('76a,b). Nevertheless, many points still remain controversial.

The hermaphroditic colonies reach sexual maturity only after having gone through several blastogenic generations. Male sexual maturity is attained first, though immature eggs are also present. Next, maturing gonads of either sex are produced in sequential generations. Seasonal sexual cycles in the wild, and succeeding cycles in the laboratory under constant temperature conditions have been described for *B. schlosseri* (Sabbadin, '55a, '60). Whether germ cells are present in the newly set colonies or during the periods or sexual inactivity and transferred to and gradually matured in the following generations

has been disputed (Pizon, 1893; Berrill, '41a,b,c). As a matter of fact, growth phase oocytes migrating through the blood stream have long been recognized and recently re-described by Izzard ('68) and Mukai and Watanabe ('76a). It is not known whether presumptive male cells are also transmitted. Even the question of germ cell origin, either from the atrial epithelium of bud primordia (Berrill, '41a,b,c) or from hemoblasts, the source of hemocytes, (Mukai and Watanabe, '76a) has not been definitively settled.

The sexual contribution of the zooids within the same blastogenic generation differs contralaterally in each zooid and between zooids derived from the right and left sides of the same parent (Sabbadin, '60; Mukai and Watanabe, '76b). It has not yet been ascertained

whether this bilateral asymmetry reflects merely different capacities of gonad growth and maturation or also of gonad production.

This paper deals with the above problems. Some preliminary results have been presented elsewhere (Sabbadin et al., '70; Sabbadin, '73).

MATERIALS AND METHODS

The Botryllus colony: structure and development

A colony of *B. schlosseri* develops from a metamorphosed larva in the form of a clone of zooids enveloped in a common tunic and ordered in star-shaped systems (fig. 9). The tunic is traversed by a vascular network connecting the animals to a marginal vessel which expands into a crown of peripheral ampullae (fig. 1). Three sequential generations of zooids will always be present: the adults and two generations of bilateral, primary and secondary, buds (fig. 1); the buds are permanently attached to the parents by an epidermal stalk conveying the blood and the nutrients (fig. 3). Zooids of the same generation are at the same developmental stage. At 18°C a weekly change of generation takes place. The regression of the adults is accompanied by the maturation of the primary buds and the initiation of a new generation from the secondary buds. At the above temperature the entire life cycle of zooids is about three weeks. It has been subdivided (Berrill, '41a; Sabbadin, '55b) into 11 stages: stages 1-6 refer to the secondary buds, stages 7-8 to the primary buds and stages 9-11 to the adults. The stages of the colony as a whole result from the combination of the stages of the coexisting generations; they can be indicated as follows: 9/7/1, 10/8/2-5, 11/8/6.

Budding is palleal: a secondary bud arises from the atrial epithelium of a primary bud, covered by the epidermis (fig. 2). The epidermis gives rise to the bud epidermis plus the connecting stalk, the atrial epithelium gives rise to all the other bud structures (Berrill, '41a). The first indication of a pair of bud primordia is a local bilateral thickening in the parental atrial epithelium (st. 1, fig. 3), which then curves into a prominent hemisphere (st. 2, fig. 4) and finally segregates as a closed vesicle overlayed by the epidermis (st. 3, fig. 5). The atrial vesicle then expands into an antero-posterior direction; the gut rudiment evaginates from its left posterior end (st. 4). Later the neural and heart rudiments appear, while two longitudinal folds initiate the subdivision

of the vesicle into a central, branchial, and two lateral, peribranchial, chambers (st. 5, figs. 6, 14); the subdivision is completed at stage 6 (fig. 7). Stage 7 marks the passage of the secondary bud to the condition of primary bud with the primordia of a new generation (fig. 3). The heart beat begins at the early stage 8. At the opening of the siphons (st. 9) the primary buds pass to the adult condition and their eggs are fertilized; embryonic development and discharge of larvae and sperm characterize stage 10. At stage 11 the adults regress and are eventually resorbed (fig. 8).

Techniques employed

Colonies from our permanent *Botryllus* cultures (Sabbadin, '55b; '60) were used. They were bred in the laboratory, attached to glass slides at the metamorphosing larval stage, then reared in aerated sea water maintained at a constant temperature of 18°C, and fed with unicellular algae. Growth, generation changes and sexual condition were constantly monitored with a dissecting microscope.

The process of colony sexualization was investigated histologically in a series of young colonies fixed periodically throughout a sequence of several blastogenic generations. Older colonies were employed in a histological study of the sexual cycle in successive generations. Accordingly, their systems of zooids were fixed individually at time intervals.

The differential bilateral development of the gonads was investigated with reference to its occurrence in each zooid and twin zooids

Fig. 1 A very young colony of *B. schlosseri* seen from the ventral side. Three coexisting generations of zooids — adults (A), primary (PB) and secondary (SB) buds — enveloped in a common tunic which shows a collecting marginal vessel (mv) and a crown of ampullae (a) branching out from it. More primary buds are present on the right than on the left side of the parents; right and left are indicated by heart (h) and gut (g) positions, respectively. × 21.

Fig. 2 Cross section of a primary bud showing secondary bud (SB) primordia arising from the atrial epithelium (ae) covered by epidermis (ep). × 220.

Fig. 3 Secondary bud (SB) primordia as a bilateral thickening of the atrial epithelium from a primary bud (PB) which is connected by an epidermal stalk (s) to the parental adult (A). Colony stage 9/7/1. × 50.

Fig. 4 The hemisphere stage of secondary buds (SB). Colony stage 10/8/2. × 50.

Fig. 5 The vesicle stage of secondary buds (SB). Colony stage 10/8/3. × 50.

Fig. 6 Secondary buds (SB) at stage 5. Colony stage 10/8/5. × 50.

Fig. 7 A regressing adult (A), a primary bud (PB), and secondary buds (SB) at stage 6. Colony stage 11/8/6. × 50.
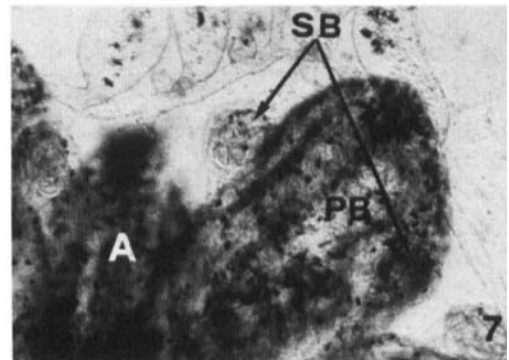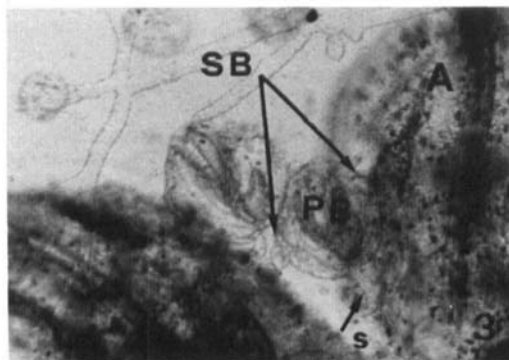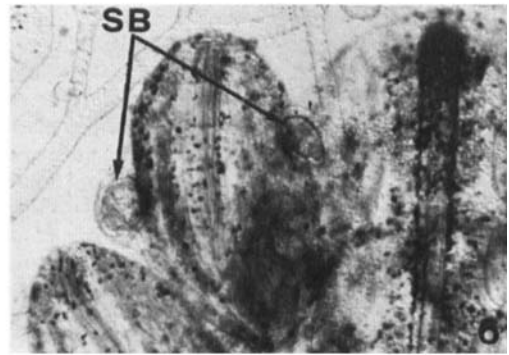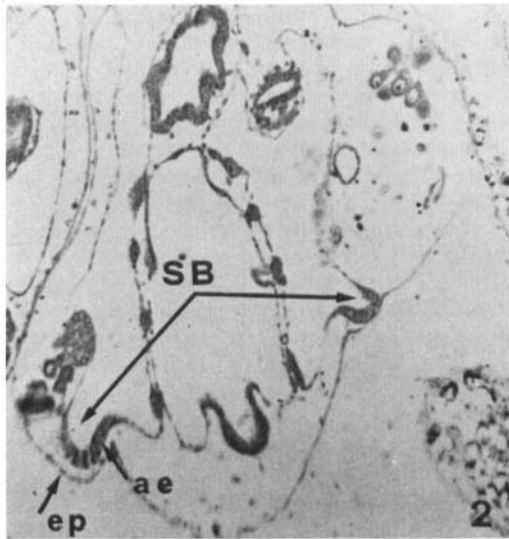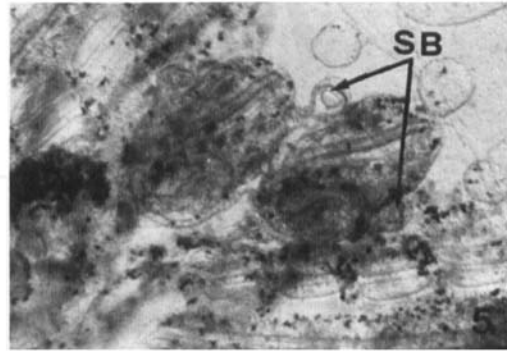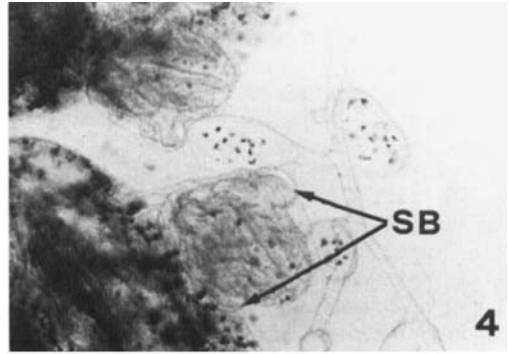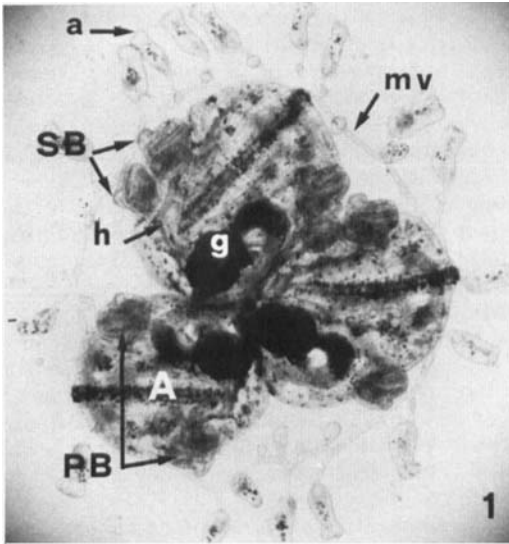
originated contralaterally from the same parent. The data were treated statistically.

The transfer of germ cells was studied by the fusion technique. It is known (Oka and Watanabe, '57; Sabbadin, '62) that if two colonies, which share at least one allele at a major histocompatibility locus, are placed side by side, the vascular ampullae of the confronting tunics then interdigitate at the contact area (fig. 10); next, apertures form at the points of contact, so that the vascular systems of the two colonies anastomose (fig. 11). A permanent parabiosis is established, which can be interrupted by cutting through the tunic at the interface between the fused colonies. During the parabiosis the individuality of the partners is maintained, since only palleal buds continue to be formed by the preexisting zooids. In fact, *B. schlosseri* does not spontaneously produce vascular buds (Milkman, '67; Sabbadin et al., '75) which, if produced during the parabiosis, might result in mosaic zooids. Therefore, if germ cells are exchanged between the parabionts, this can occur only through the blood stream. We prepared a series of parabiotic pair fusions between colonies of the opposite genotypes *AAbb* and *aaBB*. These genotypes refer to two Mendelian pigmentation genes (Sabbadin and Graziani, '67), the two couples of alleles *A* and *a*, *B* and *b* being responsible for the presence and the absence of orange pigment, and for the presence and the absence of the intersiphonal double band (fig. 9), respectively. Whereas the orange pigment cells can be transferred by blood from one to the other parabiont, this is not the case for the band which, therefore, serves as a marker of the boundaries between the two colonies when they are separated. After separation each of the two parabionts was crossed with the double recessive genotype *aabb*. Colonies phenotypically *aB* in the offspring from *AAbb*, and *Ab* in the offspring from *aaBB* were interpreted as having arisen from germ cells received from the partner during the fusion period.

RESULTS

Sexual differentiation in the colony and in the zooids

The newly set colonies persist in a condition of asexuality or sexual immaturity for several blastogenic generations; growth phase oocytes transferred through the generations as well as abortive testes become visible in vivo at specific times. On the other hand, the adult colonies are either in a condition of full sexual maturity, each generation giving rise to both eggs and sperm, or in a condition of sexual depression with the generations involved showing the same features as the younger generations. Therefore, the sexual differentiation of zooids must be considered in the context of the temporal sexual evolution of the colony as a whole. To follow these phenomena pieces of nine adult colonies were fixed over a period of time extending for two successive adult generations and involving the bud stages of one or two additional generations, and 36 young colonies were sacrificed in the interval from the first to the fourteenth generation. The histological study dealt with the sequential sexual phases in the colonies and the corresponding gonad development in their zooids.

A comparative study of gonad structure in several botryllid species, including *B. schlosseri*, has been performed by Mukai ('77) and an exhaustive description of the zooid sexual differentiation in sexually mature colonies of the allied species, *B. primigenus*, has been given by Mukai and Watanabe ('76a,b). We shall present our own data with emphasis on those points which differ from the observations and conclusions of the above authors.

In the colonies in the phase of full sexual maturity a testis and an ovary are lodged bilaterally in the mantle of adults and primary buds, between epidermis and the atrial epithelium, just posterior to the budding region (fig. 12). The testis is a coherent structure with a delimiting epithelium (figs. 17-20); the ovary is a loose structure consisting of oocytes of different sizes and stages and of a variable number of still undifferentiated cells (figs. 16, 23).

In the secondary buds a bilateral gonad blastema appears at stage 4 in the form of a clump of undifferentiated cells adhering to the atrial epithelium (figs. 13-15); its medial


Fig. 8   The regressing adults (A) possess only dextral primary buds having gonads (go) on the only left side. × 15.

Fig. 9   Two fused colonies within a common tunic. The intersiphonal double band (db) distinguishes colony A from colony B. × 6.

Fig. 10   Interdigitating ampullae at the contact area between two confronting colonies. × 15.

Fig. 11   The contact area (ca) between the colonies A and B traversed by the vascular ampullae (a) of A which have opened into the vascular system of B at confluence points (cp). × 14.

Fig. 12   Testis (t) and ovary (ov) of a primary bud (PB) behind the budding region from which secondary buds (SB) have originated. × 30.
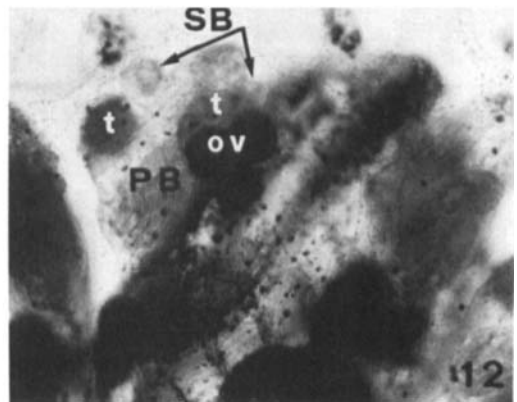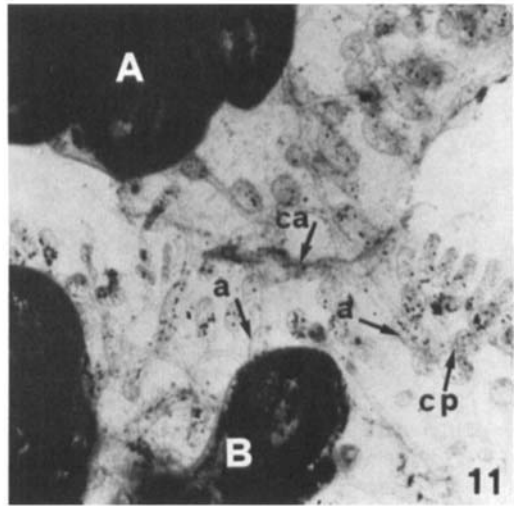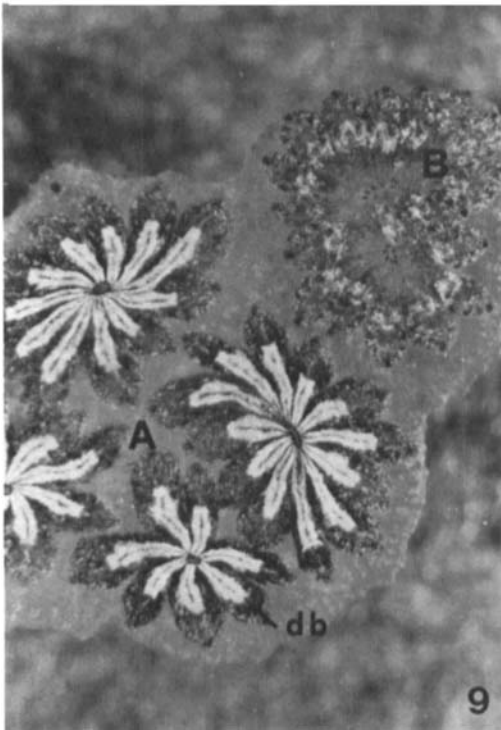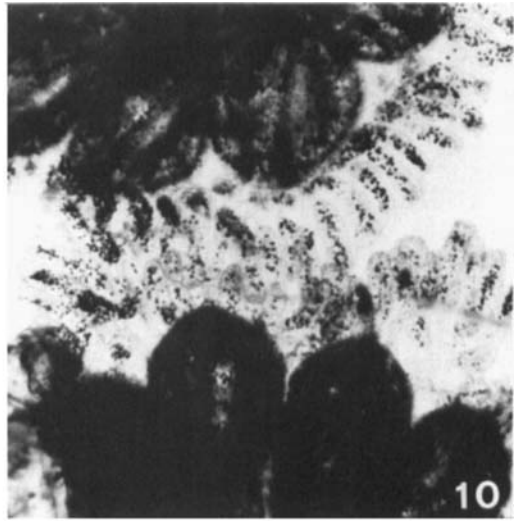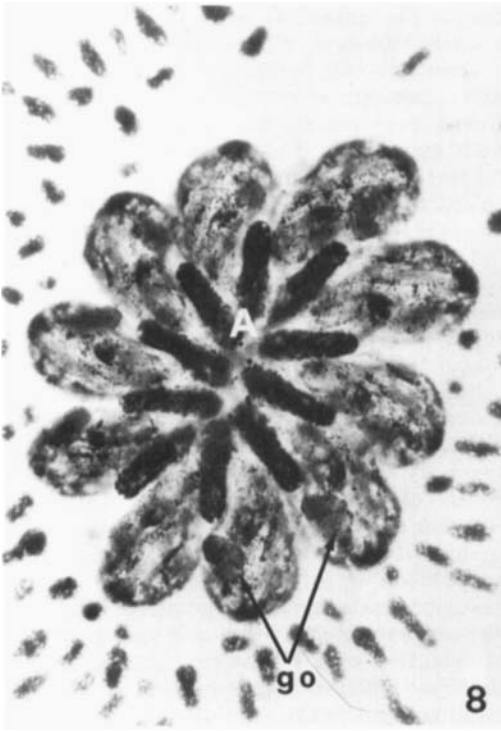
portion will give the testis, the lateral one will become the ovary. The blastema is often accompanied by growth phase oocytes transferred from the preceding generations, principally from primary buds. In the opinion of Izzard ('68) and Mukai and Watanabe ('76a,b), the blastema has also this same exogenous origin, its cells having entered the blood from the ovarian area of primary buds. According to Mukai and Watanabe, these cells would correspond to hemoblasts, the source of hemocytes. We found that the diameter of the vesicular nucleus of 16 cells from an early gonad primordium averaged 6.17 ± 0.18 μm and the nuclear diameter of 66 circulating hemoblasts averaged 3.88 ± 0.37 μm. Thus, in spite of morphological similarities, hemoblasts and germ cells represent distinct cell populations (figs. 16A-D).

The testis becomes evident as a solid mass of undifferentiated cells in the secondary buds at stage 6. Later, in the primary buds, the outer cell layer evolves into a delimiting epithelium; the testis becomes hollow during a growth period which entails the formation of a variable number of lobules in a morular shape (figs. 12, 17). The spermatogenesis begins in the primary buds at the colony stage 10/8/4 (fig. 18) and continues in the adults (fig. 19). Sperm discharge through a short spermiduct begins about two days after the buds have entered the adult stage and may continue until the zooids regress (fig. 20).

The prospective ovarian portion of the blastema differentiates earlier than the testicular one. This is best seen in the young colonies at the initiation of sexualization: in the secondary buds numerous cells are soon engaged in the meiotic prophase (fig. 21). These observations contrast with those of Mukai and Watanabe ('76a) who, having examined fully mature colonies, stated that secondary buds receive their entire complement of oocytes from primary buds and that only the latter are able to engender new oocytes to be passed to the following generation.

In the secondary buds at stage 6 a few oocytes undergo vitellogenesis. This will be completed in the primary buds (fig. 22). During growth and vitellogenesis the oocytes are enveloped by follicular sheets; the outer follicle expands into an oviduct (fig. 22) through which the egg is extruded into a brood pouch inside the peribranchial cavity, where it is fertilized when the bud opens its siphons. Pro-

togyny prevents the eggs from being self-fertilized (Milkman, '67; Sabbadin, '71).

Quiescent cells, occasional meiotic prophase and especially growth phase oocytes of different sizes persist in the ovary of primary buds and adults (figs. 19, 23). They are also found throughout the vascular network of the colony (fig. 16D), having been moved from the original location by blood.

The gonad development in the zooids from colonies with a depressed or inapparent sexuality contrasts with the development in the zooids from colonies in the phase of full sexual maturity. Within the group of the nine adult colonies examined, depressed sexuality found its expression in: (a) an enhancement of the differences in gonad development between the two body sides in each zooid and between the zooids of the same generation. These differences can be referred to as sexual bilateral asymmetry and will be considered under a separate heading; (b) absence of testes or underdeveloped testes not undergoing spermatogenesis and eventually degenerating; (c) absence of maturing eggs due to the fact that either the growth phase oocytes were unable to enter vitellogenesis or the vitellogenic oocytes were resorbed.

Within the group of the 36 young colonies examined the sexual condition varied mainly according to age and fell into one of the following five categories: (a) *asexuality* — no traces of either gonads or free germ cells, in any of the coexisting generations, in 16 colonies ranging from the first to the eighth generation; (b) *incipient sexuality* — only undifferentiated germ cells grouped into a small blastema in the secondary buds, or dispersed as free elements in the primary buds and adults of two couples of colonies, one in the sixth, the other in the ninth generation; (c) *hypofemaleness* — only female gonads with prophase and/ or growth phase oocytes and no vitellogenic oocytes in 12 colonies covering the interval from the sixth to the fourteenth generation. In one colony oocytes were present only in the

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Figs. 13, 14   Cross sections of secondary buds at stages 4 and 5, respectively, showing gonad blastemata (go) and growth phase oocytes. Figure 13 × 560; figure 14 × 410.

Fig. 15   Lateral view of a secondary bud at stage 5 showing a gonad blastema joined by immigrated oocytes. × 1,100.

Fig. 16A-D   Undifferentiated germ cells (gc) in the ovarian area of a primary bud (A), in a degenerating testis (B) and in a vascular ampulla (D), compared for the nuclear size with hemoblasts (he) (C). × 1,400.
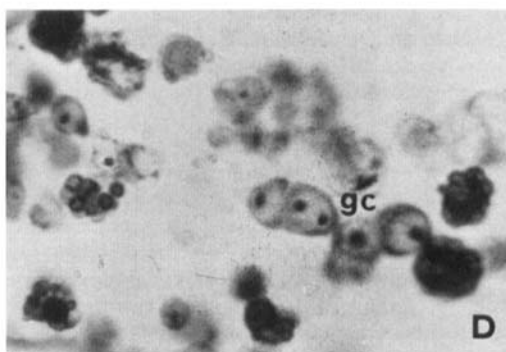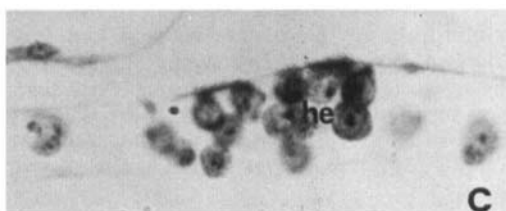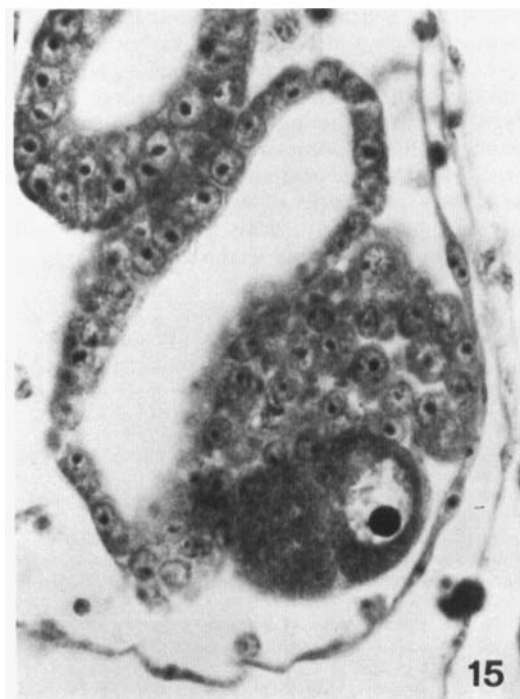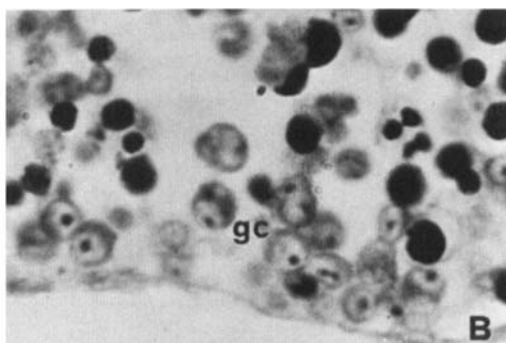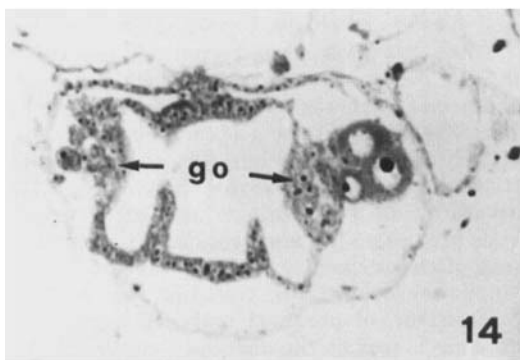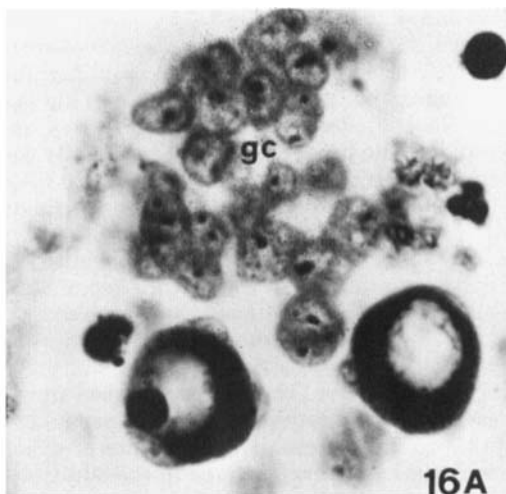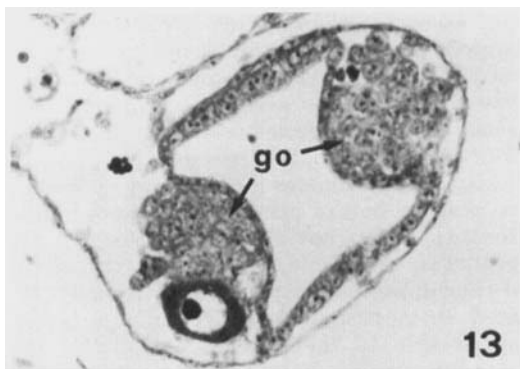
secondary buds, where they must have formed; (d) *hypofemaleness and hypomaleness* — in two colonies, which were examined for two successive adult generations, female gonads lacked vitellogenic oocytes; testes appeared at the proper time, but eventually degenerated; (e) *hypofemaleness and maleness* — spermatogenetic testes and ovaries without vitellogenic oocytes in two colonies.

The above sequence of sexual conditions, finally culminating in the maturation of both eggs and testes *(femaleness and maleness)*, seems to represent the direct sequence of phases in the acquisition of sexual maturity by young colonies. There is also reason to believe that in the reverse sequence a sexual cycle terminates. Actually, both types of hypofemaleness were encountered in the adult colonies, and some data from the experiments of parabiosis, to be reported later, suggest that germ cells might disappear from colonies during certain periods of sexual inactivity.

Sexual bilateral asymmetry

In *Botryllus* not only the viscera but also the blastogenic and sexual potentials are bilaterally asymmetrical (Sabbadin, 55b; '60; Mukai and Watanabe, '76b). In *B. schlosseri* the reversal of bilateral asymmetry affects equally these features (Sabbadin, '60) which therefore can be regarded as inherent to zooid organization.

The blastogenic potential is higher on the right side. One dextral and one sinistral bud primordium are produced and both can split into two, each giving rise to one anterior and one posterior bud. Much more posterior buds form on the right side (fig. 1), while more are resorbed on the left side. Whenever only one bud per zooid matures, it is always the dextral one (fig. 11).

The sexual potential is higher on the left side, as judged on leaving material by the presence and size of maturing gonads (fig. 11). Moreover, it differs between zooids, depending on whether they evolve from dextral or sinistral buds.

It is important to answer the question whether sexual asymmetry reflects merely different capacities for gonad growth and maturation, or also involves different capacities for gonad formation. For this purpose, we have performed a quantitative comparative study of gonad development in the different categories of zooids of the coexisting generations in the group of the nine adult colonies.

Presence and number of, (a) prophase and growth phase oocytes in secondary buds, (b) vitellogenic oocytes in primary buds, (c) larvae in adult zooids were the parameters considered in the three sequential steps of ovary formation, growth, and maturation. The corresponding parameter for testes was presence or absence in the primary buds and adults. Neither testes nor vitellogenic oocytes appeared on either side of some primary buds or, if they appeared, they subsequently degenerated. In no case did zooids exhibit testes on either side in the absence of ovaries; the opposite was often true. The testis size, as roughly expressed by the lobulation degree, was found to be always bigger on the left side.

Table 1 reports summarized data concerning the percentages of zooids of the different categories with testes and ovaries on either side. The data point to a sexual potential constantly higher on the left. They also suggest that this potential varies between zooids according to their origin: dextral primary buds prevail on the sinistral ones; the same is true of the secondary buds at stages 4-6, provided they derived from the same type, dextral or sinistral, of parental buds. However, the data are biased by the unequal contribution to the total number of zooids from colonies varying in the sexual condition, and by the fact that at stage 4 of the dextral secondary buds gonads have not yet appeared in their sinistral partners which are somewhat retarded. Therefore, in the following the statistical significance of the differences in gonad development was evaluated by comparing an equal number of adults and pairs of buds from the same parents per colony, with the sinistral secondary buds beginning with stage 4, following a random scheme.

Thirty adults and six pairs of primary buds from each of six colonies were considered with reference to ovary and testis maturation, tes-

Fig. 17 Ovary (ov) and lobulated testis (t) from a primary bud at stage 8/2. × 560.

Fig. 18 Testicular lobules at the beginning of spermatogenesis from a primary bud at stage 8/4. × 1,400.

Fig. 19 Maturing testis and residual oocytes (o) from an adult zooid. × 240.

Fig. 20 Emptying testis of an adult zooid. × 480.

Fig. 21 Lateral view of a secondary bud at stage 5 with meiotic prophase oocytes in the gonad blastema. × 1,100.

Fig. 22 Cross section of a primary bud at stage 8/4: testes (t) and a vitellogenic oocyte with follicles and oviduct (od). × 200.

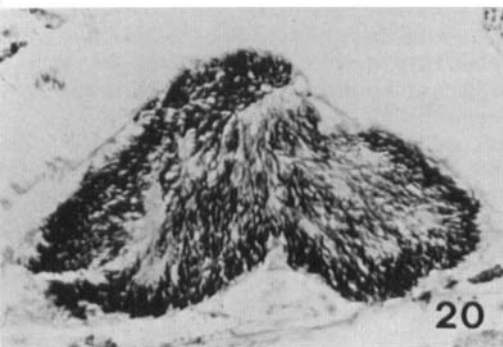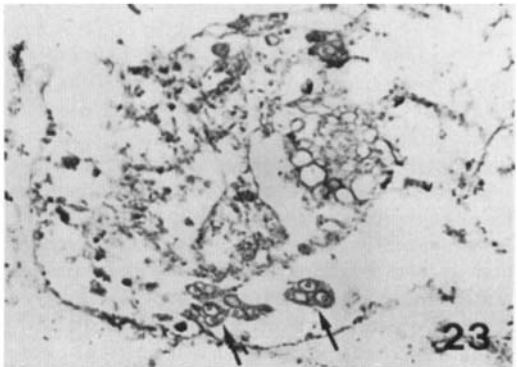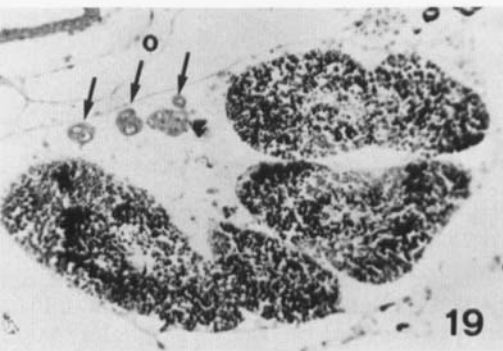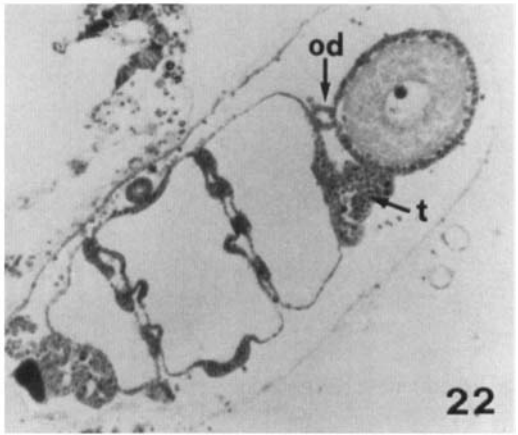Fig. 23 Residual growth phase oocytes (arrows) in a regressed adult. × 220.
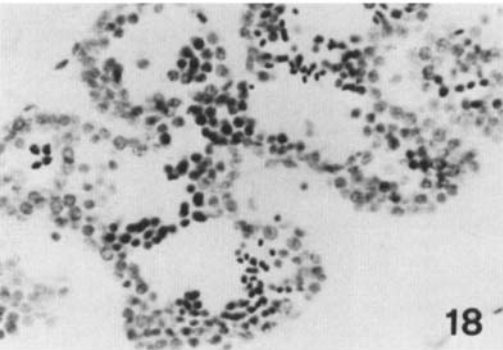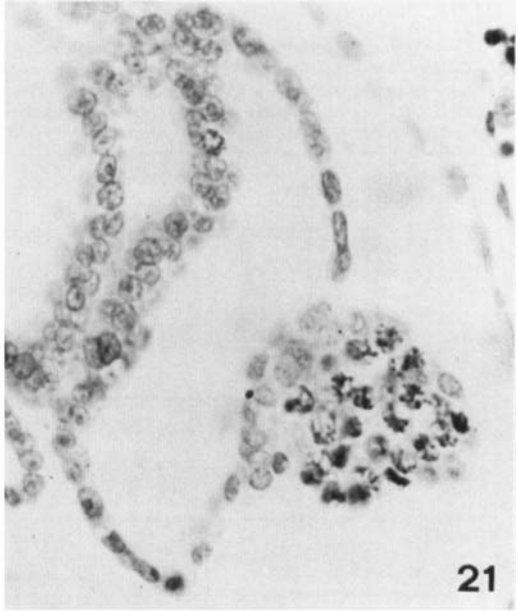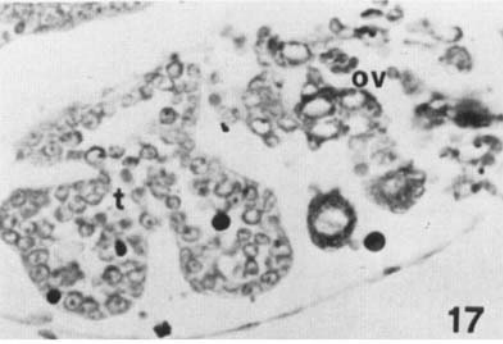
TABLE 1

Comparison of presence of contralateral testes and ovaries in the zooids of nine adult colonies. Ovary includes: larvae in the adults, vitellogenic oocytes in the primary buds, meiotic prophase and/or growth phase oocytes in the secondary buds. D,S, primary buds of either dextral or sinistral origin in the parental adult; D/D,S/D, secondary buds of either dextral or sinistral origin in the dextral primary bud; D/S,S/S, secondary buds of either dextral or sinistral origin in the sinistral primary bud

Type of zooids		No. of zooids	Testes: % zooids		Ovaries: % zooids	
			Left side	Right side	Left side	Right side
Adults		324	88	65	37	9
Primary buds:	D	215	91	59	52	19
	S	87	52	20	10	2
Secondary buds:	D/D	131			80	62
	S/D	100			29	11
	D/S	39			51	41
	S/S	30			17	7

TABLE 2

Contralateral testes in 30 adults from each of six colonies

Colony	No. of testes		
	Left side	Right side	Total
1	30	24	54
2	21	14	35
3	21	12	33
4	19	10	29
5	29	22	51
6	30	24	54
	150	106	256

Analysis of variance

Source of variation	d.f.	Sums of squares	Mean squares	F
Total	11	498.67		
Between sides	1	161.34	161.34	173.48**
Between colonies	5	332.67	66.53	71.54**
Residual	5	4.66	.93	

tis formation and growth and ovary growth. For both types of zooids highly significant differences between the two body sides were observed with respect to the presence of testes, presence of larvae or vitellogenic eggs, number of larvae or vitellogenic eggs. Two out of the six comparisons are reported in tables 2-3.

Pairs of secondary buds belonging to dextral primary buds were considered with respect to ovary formation. Four pairs of buds from each of six colonies were compared with respect to the presence of prophase and/or growth phase oocytes, and three pairs of buds from the same colonies were compared with respect to the number of growth phase oocytes. Both comparisons revealed a highly significant difference between buds, and a significant difference between sides (tables 4, 5). Most of the

secondary buds without oocytes had distinct gonad blastemata either on both sides (all the dextral buds) or on the left (a majority of sinistral buds).

From these results it is safe to conclude that the sexual asymmetry in *B. schlosseri*, besides reflecting different capacities for gonad growth and maturation, also reflects different capacities for gonad formation. It plays a basic role in the overall gonad development. From this point of view, the statement by Mukai and Watanabe ('76a) that the secondary buds receive their full complement of oocytes from preceding generations is not tenable. Actually, it would hardly be conceivable that the oocytes would choose the type of bud and the body side to which they migrate.

There might be a competition between the

TABLE 3

*Contralateral vitellogenic oocytes in six pairs (dextral and sinistral) of primary buds
from each of six colonies*

Colony	Dextral buds			Sinistral buds		
	Left side	Right side	Total	Left side	Right side	Total
1	8	5	13	0	1	1
2	3	1	4	0	0	0
3	3	0	3	2	0	2
4	2	0	2	0	0	0
5	4	2	6	2	2	4
6	4	0	4	0	0	0
	24	8	32	4	3	7

Analysis of variance

Source of variation	d.f.	Sum of squares	Mean squares	F
Total	23	97.63		
Between buds	1	26.04	26.04	21.34**
Between sides	1	12.05	12.05	9.88**
Between colonies	10	46.09	4.61	3.78
Residual	11	13.45	1.22	

TABLE 4

*Contralateral ovaries in four pairs (dextral and sinistral) of secondary buds from each of
six colonies. The secondary buds belong to dextral primary buds*

Colony	Dextral buds			Sinistral buds		
	Left side	Right side	Total	Left side	Right side	Total
1	3	1	4	1	0	1
2	4	4	8	3	1	4
3	3	4	7	2	1	3
4	4	3	7	0	0	0
5	4	4	8	4	2	6
6	4	2	6	0	0	0
	22	18	40	10	4	14

Analysis of variance

Source of variation	d.f.	Sums of squares	Mean squares	F
Total	23	58.50		
Between buds	1	28.16	28.16	53.13**
Between sides	1	4.17	4.17	7.87*
Between colonies	10	20.34	2.03	3.83*
Residual	11	5.83	0.53	

blastogenic potential, which is higher on the right side, and the gonad growth which is greater on the left side of zooids. There is no such competition between bud formation and gonad formation, since the gonads of both sexes form in the zooids long before the zooids reach the budding stage.

Germ cell recycling through successive generations

Different types of germ cells are liable to be picked up by the blood stream and recycled through the zooids of successive generations: previtellogenic oocytes and quiescent cells in the ovary; presumptive male and female components of gonad blastemata which do not evolve into testes or oocytes; possibly, cells from the gonads of regressing buds.

Germ cell transfer was experimentally established both qualitatively and quantitatively.

Selected adult colonies were used to prepare

TABLE 5

Contralateral growth phase oocytes in three pairs (dextral and sinistral) of secondary buds from each of six colonies. The secondary buds belong to dextral primary buds

Colony	Dextral buds			Sinistral buds		
	Left side	Right side	Total	Left side	Right side	Total
1	7	3	10	2	1	3
2	8	5	13	5	4	9
3	8	3	11	3	2	5
4	3	2	5	0	0	0
5	5	6	11	1	0	1
6	7	2	9	0	0	0
	38	21	59	11	7	18

Analysis of variance

Source of variation	d.f.	Sums of squares	Mean squares	F
Total	23	159.96		
Between buds	1	70.04	70.04	34.85**
Between sides	1	18.38	18.38	9.14*
Between colonies	10	49.42	4.94	2.46
Residual	11	22.12	2.01	

TABLE 6

Offspring from ex-parabionts of the opposite genotypes AAbb *and* aaBB *crossed for both sexes to the genotype* aabb. *Male and female germ cells exchanged during parabiosis result in heterochthonous offspring recognizable by the phenotypes* aB *and* Ab, *respectively*

Parental genotypes	No. of crosses	No. of offspring			
		Total		Heterochthonous	
♂ AAbb × ♀ aabb	15	478	(aB)	188	
♂ aaBB × ♀ aabb	10	492	(Ab)	185	
		970		373	(38.4%)
♀ AAbb × ♂ aabb	16	244	(aB)	36	
♀ aaBB × ♂ aabb	12	266	(Ab)	105	
		510		141	(27.6%)

22 parabiotic pairs, the two parabionts possessing opposite genotypes, *AAbb* and *aaBB*. The parabiosis duration varied from two days to a week or more. After having been separated, the parabionts were individually crossed to colonies of the genotype *aabb*. The crossers acted alternately as females and then males in each of a variable number of blastogenic generations. Thus, both male and female germ cells, exchanged between parabionts *AAbb* and *aaBB*, resulted in colonies of the phenotype *aB* in the two series of offspring from ♀ *AAbb* × ♂ *aabb* and ♂ *AAbb* × ♀ *aabb*, and in colonies phenotypically *Ab* in the offspring from ♀ *aaBB* × ♂ *aabb* and ♂ *aaBB* × ♀ *aabb*. Thirty-six out of the forty-four ex-parabionts were employed in the crosses: 17 as both females and males, 11 as females, and 8

as males. Their reproducing generations were assigned progressive numbers, number 1 indicating the youngest of the three generations which were present in the colonies at the time that parabiosis was interrupted.

The summarized data of the experiment are reported in table 6. In 16 out of 25 crosses in which ex-parabionts were used as males, and in 15 out of 28 crosses in which they were used as females, part of the offspring resulted from germ cells exchanged during the parabiosis (heterochthonous offspring). Heterochthonous offspring were still obtained from female germ cells at the fourteenth generation and from male cells at the fifteenth generation. As can be seen from table 7, in which the most significant cases are recorded, the heterochthonous to total offspring ratios varied con-

TABLE 7

Ratios of heterochthonous to total offspring from pairs of ex-parabionts. Each member was crossed for both sexes (male line, female line) to a common genotype during successive blastogenic generations. The generations have been grouped

Pairs of partners	Offspring, male line			Offspring, female line		
	Gens. 1-5	6-10	11-15	1-5	6-10	11-15
1.1	141/146	0/9		1/3	0/46	
.2	0/12					
2.1	77/124			0/4	2/2	
.2	0/19	0/4	0/8	0/11	1/1	
3.1	41/46	2/2		0/3	3/3	
.2	0/9				0/3	
4.1	0/4	21/28		3/23	42/44	
.2	2/13	0/8	0/7	6/39	0/6	0/2
5.1	1/2	9/9		0/23		
.2	2/2					
6.1	0/11					
.2				17/17	35/35	
7.1	1/1	0/3	1/7	0/8	0/3	2/2
8.1				2/2	14/14	
9.1	0/81	11/11		0/18	1/1	
10.1	20/20			0/11	3/3	

siderably between ex-parabionts and their succeeding generations in both male and female lines. The most interesting are those cases in which the entire offspring were heterochthonous. The variability of the ratios, that may have partly depended on different parabiosis durations which allowed different amounts of germ cell exchange, for the most part ought to be ascribed to the degree of sexualization varying between the parabionts and their sequential generations. Actually, sexual reproduction underwent periods of reduction and stasis in the colonies used in the experiment. In such instances where autochthonous germ cells were very few or absent, heterochthonous cells must have contributed the majority or even all of the gametes.

These results show that in the colonies of *B. schlosseri* there is, in addition to the well known circulation of oocytes, a circulation of already determined primordial germ cells able to evolve into gemetes of either sex. We have confirmed that these cells do not correspond to uncommitted hemoblasts. Actually, it is highly improbable that only heterochthonous hemoblasts were involved in the formation of gonads in those generations that, long after the parabiosis interruption, yielded only heterochthonous offspring.

DISCUSSION

The sexual differentiation of *B. schlosseri* was considered under two aspects: (a) the sexualization of the colony as a whole throughout the sequence of its blastogenic generations; (b) the sexual contribution of the different kinds of zooids within each generation.

We did not find any trace of gonad blastemata in the first generations, nor did Berrill ('41a,b,c). This is the departure condition of asexuality, simply referred to as absence of testes and recognizable oocytes by Krohn (1869), Della Valle (1881) and Hjort (1893). There is a general agreement that after this phase some generations are involved in a condition of hyposexuality with germ cells which do not reach maturity. According to our observations, hyposexuality begins with the appearance of primordial germ cells (incipient sexuality), continues with the incomplete differentiation of ovaries in the absence of testes (hypofemaleness) and the incomplete differentiation of both ovaries and testes (hypofemaleness and hypomaleness). Male sexual maturity (hypofemaleness and maleness) occurs prior to the appearance of generations maturing both testes and ovaries (femaleness and maleness), as noticed also by Pizon (1893), Berrill (41a,b,c) and Mukai and Watanabe ('76a). The colonies reared in our laboratory under constant temperature conditions undergo successive sexual cycles. Each cycle possibly contains the whole sequence of the above sexual phases from initiation through culmination followed by the obverse of this sequence.

The sexual contribution of the zooids belonging to the same blastogenic generation

is strongly affected by two factors: sexual bilateral asymmetry; immigration of germ cells from the preceding generations.

The sexual asymmetry, that in other polystyelid ascidians may take the form of a complete separation of male and female gonads on the two body sides (Berrill, '48; Newberry, '68), in *Botryllus* is of a mere quantitative type. It takes the form of an unequal gonad development both contralaterally in each zooid and in zooids of a different, dextral or sinistral, origin. The differences are noticeable from the moment at which gonad blastemata appear. Actually, the blastemata are more often absent and their size is often smaller on the right side and in sinistral buds. Accordingly, on the right side and in sinistral buds, more often than on the left side and in dextral buds, maturing testes and eggs do not form or they are less developed or less numerous. The differences between zooids is just an aspect of their different developmental capacities, the belated and smaller sinistral buds suffering competition with the dextral ones (Sabbadin, '60). The gonadogenic potential becomes higher on the right side than on the left when the bilateral asymmetry of viscera is experimentally reversed (Sabbadin, '60). It should be correlated with the different morphogenetic processes occurring on the two sides.

The recycling of germ cells involves not only the oocytes but also undifferentiated germ cells. Our parabiosis experiments showed that the transferred cells may persist for many generations as circulating elements before maturing as sperm or eggs. The transfer of germ cells between succeeding generations allows the maturation of a number of germ cells which otherwise would be lost, as is the case of those polystyelid species lacking vascular connections between zooids (Berrill, '48; Newberry, '68). On the other hand, this phenomenon may lead to an underestimate of the real sexual potential of the single zooids. For instance, in the opinion of Mukai and Watanabe ('76a) the contribution to the ovary formation by the youngest of the three generations in the colony would principally consist in growing the oocytes immigrated from the parents; only much later, after having produced its own buds, this generation would form new oocytes destined for transfer to the next generation. It is true that at the verge of a sexual cycle many growth phase oocytes can

migrate into the young buds; there they can strongly inhibit the formation of new oocytes, as a result of competition for the same nutrients, a process which is also at work in limiting the number of oocytes undergoing and completing vitellogenesis. Nevertheless, we showed that a greater number of oocytes on the left than on the right side characterize the secondary buds, and this cannot be referred to as a differential migration into the two sides. Actually, the gonad blastemata of the secondary buds form precociously both meiotic prophase oocytes, as described also by Izzard ('68), and growth phase oocytes. This is better seen in the colonies at the beginning of a sexual cycle. In principle, we do not consider untenable the idea that under optimal conditions both male and female gonads can form and develop to full maturity in one and the same generation. This idea has been unduly generalized by Berrill ('41a,b,c), who denies the germ cell migration.

According to Berrill ('41a,b,c) germ cells already determined with respect to either sex would segregate from the atrial epithelium of the secondary buds within a limited perio- in three successive waves, each wave at a specific time: first, female cells capable of maturation; next, presumptive abortive eggs plus male cells; the latter are destined to form the testes together with a third wave of purely male cells. Since the germ cells within the atrial epithelium are only a small fraction of the total number of cells, their presence and quality would depend on the size already achieved by the bud at the moment of their potential segregation; this size increases in the course of both bud development and colony growth. Accordingly, no germ cells would be segregated in the to small buds of the first generations; then, for a few generations, buds of intermediate size would produce only the third or both the third and the second wave of germ cells that would form only testes or testes plus abortive eggs; only in older colonies there are buds of maximal size with all the three waves of cells segregating and producing both testes and maturing eggs. Our data are in agreement with the hypothesis of Berrill insofar as they confirm that the very young colonies are asexual and that male maturity is attained first. However, it must be pointed out that oocytes appear in the young colonies prior to the testes and that adult colonies undergo new sexual cycles, possibly of

the same sequence of phases as the first cycle. Therefore, we would consider the presence or absence of gonad blastemata as not being strictly dependent on bud size. On the other hand, there is no evidence of the primordial germ cells being predetermined as female or male. The determination might just as well be the result of different local influences on the medial and lateral portions of the gonad blastema which represent the presumptive testis and the presumptive ovary, respectively. For instance, the lateral portion is more exposed to the blood that circulates between epidermis and the atrial epithelium to which the blastema adheres. As a matter of fact, a thin blastema gives rise only to an ovary, and no testis is formed without an accompanying ovary. The absence of testis does not necessarily mean absence of male cells, since a testicular structure needs a minimal critical cell number to be expressed. Different physiological conditions acting as sexual determinants on sexually uncommited gonad primordia have also been postulated by Newberry ('68) in *Distomus variolosus*.

In contrast to Berrill, Izzard ('68) and Mukai and Watanabe ('76a) claim that there is no evidence of germ cells segregating from the atrial epithelium of secondary buds. The gonad blastemata in these buds would consist of cells immigrating from the parental generation in the form of hemoblasts, according to Mukai and Watanabe, or in the form of elements cytologically similar to early spermatogonia and possibly representing a primordial germ cell stock, according to Izzard. We have demonstrated that such a stock does exist in *Botryllus* as a specific population of cells differing in size from hemoblasts. Moreover, the parabiosis experiments showed that ex-parabionts in certain generations produced only heterochthonous offspring from germ cells received from the partners. This result cannot be explained by assuming that the transmitted cells were nothing more than uncommitted hemoblasts, the germinalization of which was delayed. It must be assumed that the cells belonged to an already determined germ line.

In principle, we have no objection to the thesis that the primordial cells of the blood line might have the ability to transform into germ cells, as has also been postulated for other polystyelid ascidians such as *Distomus* (Newberry, '68) and *Symplegma* (Sugimoto

and Nakauchi, '74). However, as far as *Botryllus* is concerned, we do not think that at present the evidence for this thesis is more substantial than for the thesis which postulates that germ cells segregate from the atrial epithelium of buds. Further experiments are needed in order to decide which hypothesis for the two possible sources of germ cells is valid.

LITERATURE CITED

Berrill, N. J. 1941a The development of the bud in *Botryllus*. Biol. Bull., *80:* 169-184.
———— 1941b Size and morphogenesis in the bud of *Botryllus*. Biol. Bull., *80:* 185-193.
———— 1941c Spatial and temporal growth pattern in colonial organisms. Growth, *5* (Suppl.): 89-111.
———— 1948 The gonads, larvae, and budding of the polystyelid ascidians *Stolonica* and *Distomus*. J. Mar. Biol. Ass. U. K., *27:* 633-650.
Della Valle, A. 1881 Nuove contribuzioni alla storia naturale delle Ascidie composte del Golfo di Napoli. Atti Accad. Lincei, *10:* 431-498.
Hjort, J. 1893 Uber den Entwicklungscyclus der zusammengesetzen Ascidien. Mitth. Zool. Stat. Neapel, *10:* 584-617.
Izzard, C. S. 1968 Migration of germ cells through successive generations of pallial buds in *Botryllus schlosseri*. Biol. Bull., *135:* 424.
Krohn, A. 1869 Uber die Fortpflanzungsverhaltnisse bei den Botrylliden. Arch. Naturgesch. (Berlin) *35:* 189-196.
Milkman, R. 1967 Genetic and developmental studies on *Botryllus schlosseri*. Biol. Bull., *132:* 229-243.
Mukai, H. 1977 Comparative studies on the structure of reproductive organs of four Botryllid species. J. Morph., *152:* 363-370.
Mukai, H., and H. Watanabe 1976a Studies on the formation of germ cells in a compound ascidian, *Botryllus primigenus* Oka. J. Morph., *148:* 337-362.
———— 1976b Relation between sexual and asexual reproduction in the compound ascidian, *Botryllus primigenus*. Sci. Rep. Fac. Educ. Gunma University, *25:* 61-79.
Newberry, A. T. 1968 The gonads and sexual cycle of the polystyelid ascidian *Distomus variolosus* Gaertner. J. Morph., *126:* 123-137.
Oka, H., and H. Watanabe 1957 Colony-specificity in compound ascidians as tested by fusion experiments. Proc. Imp. Acad. Japan, *33:* 657-659.
Pizon, A. 1893 Histoire de la blastogénèse chez les Botryllides. Ann. Sc. Nat., Zool., *14* (7): 1-386.
Sabbadin, A. 1955a Il ciclo biologico di *Botryllus schlosseri* (Pallas) nella Laguna di Venezia. Arch. Ocean. Limn., *10:* 217-230.
———— 1955b Osservazioni sullo sviluppo, l'accrescimento e la riproduzione di *Botryllus schlosseri* in condizioni di laboratorio. Boll. Zool., *22:* 243-263.
———— 1960 Ulteriori notizie sull'allevamento e sulla

biologia dei Botrilli in condizioni di laboratorio. Arch. Ocean. Limn., *12:* 97-107.

——— 1962 Le basi genetiche della capacità di fusione fra colonie in *Botryllus schlosseri*. Rend. Acc. Naz. Lincei, *32:* 1031-1035.

——— 1971 Self- and cross-fertilization in the compound ascidian *Botryllus schlosseri*. Devel. Biol., *24:* 379-391.

——— 1973 Recherches expérimentales sur l'ascidie coloniale *Botryllus schlosseri*. Bull. Soc. Zool. France, *98:* 417-434.

Sabbadin, A., and G. Graziani 1967 New data on the inheritance of pigments and pigmentation patterns in the colonial ascidian *Botryllus schlosseri* (Pallas). Riv. Biol., *60:* 581-598.

Sabbadin, A., G. Cavalieri and L. Viscovich 1970 Trasmissione delle cellule germinali per via sanguigna nella colonia di *Botryllus schlosseri* (Ascidiacea). Acta Embryol. Exp., *1970:* 198-199.

Sabbadin, A., G. Zaniolo and F. Majone 1975 Determination of polarity and bilateral asymmetry in palleal and vascular buds of the ascidian *Botryllus schlosseri*. Devel. Biol., *46:* 79-87.

Sugimoto, K., and M. Nakauchi 1974 Budding, sexual reproduction, and degeneration in the colonial ascidian, *Symplegma reptans*. Biol. Bull., *147:* 213-226.