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An improved diet for inland broodstock and the establishment of an inbred line from *Botryllus schlosseri*, a colonial sea squirt (Ascidiacea)

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Abstract – Improved inland culturing methods for marine invertebrates are the most important prerequisite for the establishment of inbred lines. Here, we study the effects of improved diet on growth rates, survivorship and reproductive activities of the colonial urochordate *Botryllus schlosseri*, an important model species in a variety of scientific disciplines. Six long-term experiments (6–8 weeks each; 3–8 treatments per experiment) were conducted in an attempt to evaluate 10 different food types alone and in various combinations. We took a hierarchical approach in which some food types were contrasted and the results were used to design the next experimental set with new food types. A mixture of at least two types of diets was superior to any monotonous diet examined. Results were also characterized by high variability between colonies of the same hatch in any studied life history aspect, by the appearance of reproductive sterile colonies in the cultures, high survivorship, fast growing and long-lived colonies. The improved maintenance protocols enabled us to develop the first long-lived (> 6.5 years) inbred line of *Botryllus* with four successive generations of self-crossed pedigree animals. © Ifremer/Elsevier, Paris

***Botryllus schlosseri* / nutrition / inbred line / mariculture / tunicates**

Résumé – Une alimentation améliorée pour la culture et l'établissement d'une lignée génétique de *Botryllus schlosseri*, une ascidie coloniale (Ascidiacea). L'amélioration des méthodes de cultures d'invertébrés marins est la condition préalable la plus importante pour l'établissement d'un élevage. Nous étudions ici les effets d'une alimentation améliorée sur le taux de croissance, la survie et les activités reproductrices de l'urochordé colonial *Botryllus schlosseri*, une espèce-modèle importante dans un grand nombre de disciplines scientifiques. Six expériences de longue durée (6–8 semaines chacune ; 3–8 traitements par expérience) sont conduites afin d'évaluer dix types d'aliments différents, fournis seuls ou de façon combinée. Dans une approche hiérarchisée, plusieurs types d'aliments ont été confrontés et les résultats ont été utilisés pour établir la série expérimentale suivante avec de nouveaux aliments. Un mélange d'au moins deux types de régimes alimentaires est supérieur à tous les aliments considérés individuellement. Les résultats sont aussi caractérisés par une grande variabilité selon les types d'aliments dans les colonies issues d'une même éclosion et dans tous les aspects étudiés, par l'apparition de colonies stériles dans les cultures, un taux de survie élevé, une croissance rapide et des colonies à durée de vie longue. L'amélioration des protocoles de culture nous a permis de développer les premières cultures à longue durée de vie (> 6,5 ans) de *Botryllus* avec quatre générations successives d'animaux croisés dans une même descendance. © Ifremer/Elsevier, Paris

***Botryllus schlosseri* / nutrition / lignée génétique / mariculture / tuniciers**

1. INTRODUCTION

The colonial tunicate *Botryllus schlosseri* (Chordata; Ascidiacea) a cosmopolitan inhabitant of shallow water-hard substrata [2, 23, 37, 38] has become increasingly important as a model experimental species for a variety of biological fields [3, 18]. This group of organisms, one of the most advanced deuterostome invertebrates, has been subjected to studies in the fields of immunology, physiology, ecology, genetics, developmental biology, aging [literature cited in 3, 18, 29–31], and more. However, studies on these organisms are hampered by the facts that: (1) defined genetic stocks are not available for long-term research; (2) there is as yet no efficient laboratory methodology to completely circumvent the need for sporadic collec-

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tions of colonies from the field; and (3) all collecting protocols suffer from seasonal availability of colonies, genetic variations and problems in laboratory acclimatization.

Colonies of *Botryllus* are encrusting organisms which attach in nature to rocks, algae, other organisms, floats, ship bottoms, and a variety of shallow water artificial objects. Each colony bears up to several hundreds of morphologically identical units (zooids) embedded within a translucent gelatinous matrix, the tunic. All zooids in a single colony are parabiosed to each other through a ramifying network of blood vessels which bear, in the periphery of the colony, sausage-like blind enlargements (vascular ampullae). All zooids of a single colony are of identical genetic constitution formed by very complex and highly synchronized weekly cycles of asexual buddings, each called a blastogenesis. When a colony is split in nature or under experimental procedures into two or more fragments, each subclone usually continues to grow independently to form a new large isolated colony. This enables a simultaneous study on different subclones of the same genet, one of the advantages of working with these colonial organisms.

Although this group of organisms is common in nature, tunicates, in general, and botryllid ascidians, in particular, are not always easily cultured inland or in laboratory conditions. Several studies dealt with laboratory/inland culturing of tunicates, in general [1, 2, 7, 9, 13, 15, 21, 22, 24, 25], or specifically with the group of botryllid ascidians [1, 3, 4, 9, 14, 18, 27, 28, 30, 31, 33, 34]. However, there is no inbred line or defined stock of any urochordate species available for biological analyses at the present time. Boyd et al. [3] have outlined fundamental laboratory constructions for obtaining *Botryllus* colonies with various life-cycle characteristics for different experimental needs. All relevant studies have revealed that only improvement of existing techniques [3, 18, 33] will be most beneficial for successful establishment of long-term defined genetic stocks of *Botryllus* in the laboratory.

The present study focuses on the improvement of the artificial diet for culturing *Botryllus schlosseri* colonies. A better feeding protocol has been developed and further suggestions for additional amelioration are discussed. A direct result from these culture improvement methods and direct proof for their applicability, is the first development of long-term (almost 7 years) culture of an inbred line of colonies (5 successive generations). In this case, we developed a line of colonies important for immunological studies, since they are homozygotic for their fusibility locus [28, 30, 39].

2. MATERIALS AND METHODS

2.1. Experimental procedures

Six sets of laboratory experiments (6–8 weeks each) examining 3–8 different diets each (*table I*) on 562 *B. schlosseri* colonies, were performed. We took a hierarchical approach in which several food types were contrasted in a specific set of experiments and the results were used to design the next set of experiments with new food types. Therefore, each set of diet experiments was carried out on different batches of animals originating from different colonies representing variations in life history patterns and physiological characteristics, such as growth rates, survivorship patterns and reproductive activities, as recorded in other *Botryllus* populations [40]. This resulted in very limited comparisons between different sets of experiments. However, a diet which improved animal conditions independently in more than one set of experiments was considered as a genuine improvement to the laboratory culture of *Botryllus* colonies.

The 6 sets of experiments (*table I*) were divided into two groups: experiments performed on small, about one-month old colonies (Experiments 1–3); and those performed on oozooids about 1 week after settlement (Experiments 4–6).

Table I. Diet regimen in *Botryllus schlosseri* inland cultures.

Exp. Set	Duration (weeks)	No. of tanks	Diet in tank no. (food type; mL)*							
			1	2	3	4	5	6	7	8
1	6	3	In; 4	In; 2	In+Al; 2+2					
2	7	5	Ha; 4	Ap; 4	Ra; 4	Li; 4	In; 4			
3	7	4	Ap; 2	Ra; 4	Ap+Ra; 1+2	Ap+Al; 1+2				
4	8	8	Rc; 4	Rb; 4	Ra; 4	Rb+Ca; 2+2	Rb+Ap;2+2	Rb+Ap+Ca;1.5+1.5+1	Al;4	Ca;4
5	7	6	Rc;4	Rb; 4	Rb; 4	Rb+Ap;2+2	Rb+Ap+Ca;1.5+1.5+1	Va;4		
6	7	7	Rb+Ap;2+2	Rb+Ap;3+1	Rb+Ap;3+1	Rb+Ap;1+3	Rb+Ap;2.3+2	Rb+Ap; 1.7+2	Rb+Ap+In; 1.5+1.5+1	

* Food type: Al = Mixture of laboratory-raised unicellular algae; Ap = Artificial plankton; Ca = Commercial food, dried algae; Ha = Hatchfry encapsulation; In = Invertfood; Li = Liquifry Marine; Ra to Rc = Freeze-dried rotifers (a = laboratory raised, algal fed; b = pond raised, small strain; c = pond raised, large strain); Va = Daily variable diet on a weekly basis: Al, Al, Ra, Al, Ra, Al, In.

2.2. Animals

Sexually matured colonies of *Botryllus schlosseri* were collected from the Monterey marina, CA, USA [4]. Hatched larvae were settled on settlement slides and collected, as described by Rinkevich and Weissman [28]. The settled oozooids were shipped to Haifa (Israel) in insulated containers under constant temperature (18–20 °C). Colonies were kept in the laboratory in 17 L standing glass tanks (up to 20 colonies/tank) attached to 5.0 by 7.5 cm glass slides, one colony per slide, suspended vertically in slots of glass staining racks. Water was changed three times/week. Animals were fed once daily with commercial food, Liquifry Marine (Interpet Ltd., Dorkig, U.K.), as previously described [3], and subjected to 12:12 h light-dark regimen. The water in each tank was aerated by bubbles from an air stone and was maintained at 20 °C in a temperature-controlled culture room. Colonies were cleaned once a week by a soft, small brush to remove debris and fouling organisms.

For the purpose of the diet experiments, we used young settled offspring (oozooids) F1 and F2 of the wild collected colonies that were maintained as described above. Many of the oozooids died within 1–2 weeks after settlement. For the experiments, we chose only "healthy" offspring (with extended ampullae, transparent tunic matrix, functionally filtering zooids, and with energetic blood cells movement). These progenies were carefully detached from the settlement slides by small pieces of razor blades, allowed to be fastened to new slides, one animal per glass slide, and were acclimatized for at least one week before transferring them to the experimental tanks. In some of the experiments, one month old colonies were used. Colonies that were injured were removed from the experiments. Animals in the diet experiments were maintained as described above (10–20 colonies/aquarium) and fed once daily with various food types. Food particles were evenly distributed in the tanks by the aid of air bubbles produced by air stones.

2.3. Diets

Three different groups of diets, each one consisting of several types of foods, were tested: commercial foods, unicellular algae and freeze-dried rotifers. Commercial foods used were: Invertfood (Waterlife Research Ltd., UK), dilution 1:2; Hatchfry encapsulation 50–150 µm size (Argent Chemical Lab., Redmond, WA, USA), 7.5×10^{-3} g·mL^{-1}; Artificial plankton (Argent Chemical Lab), 7.5×10^{-3} g·mL^{-1}; Algal 161 (Cylsys, UK), dried algae, 7.5×10^{-3} g·mL^{-1}. Nutritional values and food compositions were done according to the manufacturer's designations. Unicellular algae used was composed of a mixture of 3 laboratory raised algae (final concentration 7.5×10^{7} g·mL^{-1}) including *Nannochloropsis* sp., *Dunaliella salina* and *Isochrysis galbana*. The chemical compositions and nutritional values of these microalgae can be physiologically manipulated and several studies have demonstrated that these algae carry high chemical quality for various applications in aquaculture hatcheries. Protocols for culturing algae and their nutritional values are: *Nannochloropsis* sp. [36], *Dunaliella salina* [8], *Isochrysis galbana* [35]. Freeze-dried rotifers were *Brachionus plicatilis*, 7.5×10^{-3} g·mL^{-1}. Three types of rotifer diets were used: (1) laboratory raised, algal fed (50 mg dry weight *Nannochloropsis* sp. per 10^6 rotifers), mixture of small and large strains (Japanese and Eilat strains, respectively); (2) pond-cultured, large Eilat strain (adult females, lorica length 170–200 µm, semi-continuous culture in 30 m^3 ponds, 150–300 rotifers·mL^{-1}, 26–28 °C, yeast fed, 0.33 g dry yeast·10^{-6} rotifers); (3) pond-cultured, small and large Eilat strain, conditions as above (details on rotifer cultures in [17]).

2.4. Animal observations

Colonies were observed once a week under a binocular dissecting microscope. We documented the number of zooids and primary buds, blastogenic cycle [20], the reproductive state and the general health of each colony. The 'health index' was constructed according to the norms of: bad (1 point), fair (2 points), good (3 points) and very good (4 points) which reflects the status of the following 5 morphological characteristics: ampullae expansion vs. construction, blood flow rates between systems, accumulation of pigment cells in zooids and ampullae, synchronization in blastogenesis and transparency of the tunic matrix. Each morphological characteristic was ranked from 1 to 4 and the average value for all 5 characteristics served as the health index for a specific colony, during a specific observation. Although semi-qualitative by nature, the 'health index' for each colony was independently assigned in the first experiments by two observers. Both evaluations were identical. Statistical analyses were performed with a SAS/STAT Program (Vol. 2, Version 6, 1990). For comparisons of colonies between different tanks within the same experiment, we employed ANOVA tests (on ln transformation for the number of zooids) followed by Duncan's Multiple Range tests, *t*-tests were performed for comparisons between reproductive activities.

To develop an inbred line of *B. schlosseri* one colony (no. M 1965) that hatched in the laboratory (February 1991) from a wild colony collected in the Monterey marina, California, was chosen. This colony grew well in the laboratory under the conditions mentioned above, reproduced and served as a founder for inbred lines of laboratory raised animals. We initiated this line by self-crossing of isolated ramets, where sperm from one ramet fertilized eggs of another ramet, in the absence of competing sperm. This was performed by isolation of these ramets within different aquaria for at least 2 weeks before the hatched offspring were collected. The fastest growing and health-

iest offspring colonies were used to produce the next generation of the inbred line.

3. RESULTS

3.1. Feeding experiments

The use of commercial food alone or with unicellular algae resulted in an average duplication of colony size within 6 weeks (Exp. tanks 1, 3; *table II*). This experiment reveals the significant effect of food concentration ($P < 0.05$) on colony growth (*table II*) as

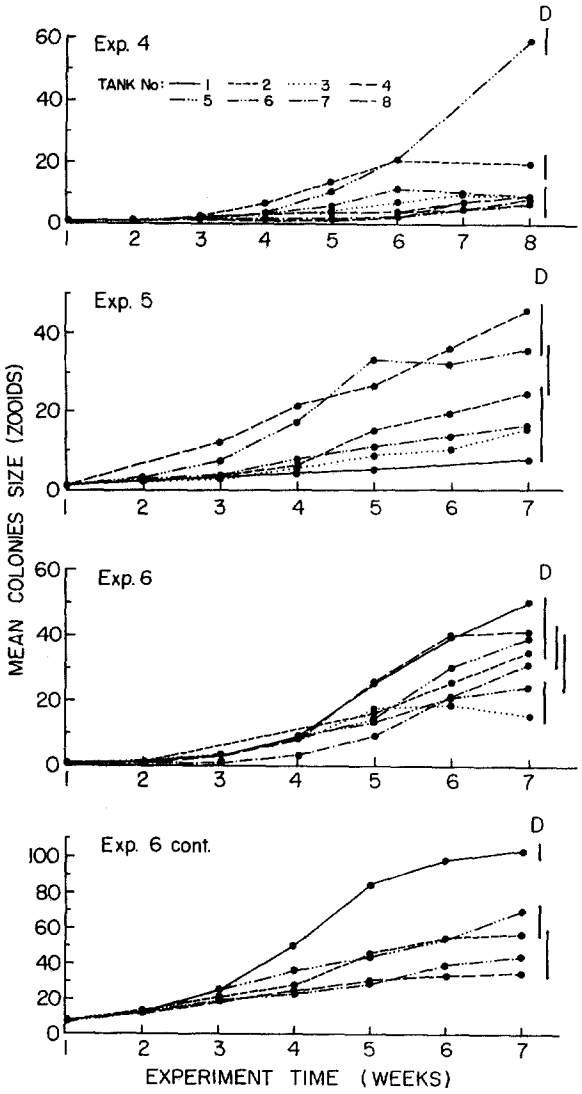

Figure 1. Average sizes of colonies in each one of the dietary-treatment tanks for the experiments started with oozooid-size colonies: (nos. 4–6), and for the follow up, continued Exp. 6. Detailed dietary regimen for exps 4–6 are in *table I;* continued Exp. 6, see text. D = Duncan multiple range tests carried out on ANOVA. Grouping conjointly on the same line are not significantly different ($P > 0.05$).

described for other sedentary filter feeders [11, 12, 32]. None of the colonies in this experiment was sexually reproductive during the experiment although survivorship in the two aquaria of the high food density was high (*table III*). This type of food was used as the basic, control type of diet in experiment 2 (*table I*), but was the least preferred diet (*table II*). Feeding with Liquifry Marine, a commercial food, yielded the highest colony average size (× 17.5 increase of size; tank 4) although two other diets (artificial plankton and rotifers) also showed good results. This experiment, as all others, is characterized by high variations in growth rates within tanks. Different colonies ranged in sizes from 2–297 zooids, a typical characteristic life history pattern of *B. schlosseri* [3, 40]. Exp. 3 concentrated on the other promising diets: artificial plankton and rotifers (*table II*). Their mixture or a mixture of artificial plankton with algae gave the best growth rate (up to a colony size of 199 zooids). Since several types of rotifers were available, we tested their effect on growth rates of young oozooids in Exp. 4. A significantly higher growth rate was recorded in tank no. 5 where a mixture of food was used (*figure 1, table III*). This possible superiority of food mixture as compared to a routine, monotonous diet was further analysed in Exp. 5 (*tables I, II; figure 1*), and again, both tanks supplied with food mixture yielded the best results. Since the combination of freeze-dried rotifers (pond cultured, small strain, yeast fed) with commercial artificial plankton (Argent Chemical Lab, *table I*), gave the best results in both Exp. 4 and 5 (*figure 1; table II*), in Exp. no. 6 different combinations of these diets were tested (*table I, figure 1*). Following the 7 weeks of this experiment, the diet mixtures of rotifers (pond cultured, small strains, yeast fed) and artificial plankton, in combinations: 2 + 2 (2 aquaria), 1 + 3, 2.3 + 2, respectively, showed the best results. To support this outcome and to evaluate the impact of other possible unrelated factors on growth rates (such as different stress conditions), we sampled at the end of Exp. 6 a single system of approximately 8–10 zooids from each colony residing in tanks 1, 2, 4, 5, 6 (the 4 best performing aquaria and an intermediate aquarium, covering all ranges of the diet combinations around the ratio of 1:1) for a follow-up experiment lasting 7 more weeks (Exp. 6 cont.). This experiment was also carried out in other tanks but under the same diet conditions as before (*figure 1*). The rotifer and artificial plankton mixture ratio of 1:1 (2 ml of each type food per aquarium, per day) was found to be the best diet combination although the average growth rate in these aquaria (nos. 1 and 2) varied significantly ($P < 0.05$, *figure 1*). In all sets of experiments, especially in experiments 4–6 (*table II*), growth rates of offspring from the same cohort, within the same aquarium and under seemingly identical environmental conditions, were characterized by high variation.

Survivorship of colonies was usually high (≥ 80 % survivorship in 29 out of 33 aquaria; no more than one

Table II. Colony sizes at the beginning and end of culture experiments.

Exp.	Duration (wk.)	Exp. phase	Average colony size and size ranges (zooids) within tank no*							
			1	2	3	4	5	6	7	8
1	6	beg.	19.5 ± 16.5	17.7 ± 7.8	15.6 ± 7.9					
			8–64	5–33	3–34					
		end	37.7 ± 29.4	12.5 ± 7.9	33.4 ± 27.7					
			10–108	5–27	6–88					
2	7	beg.	7.9 ± 4.7	8.6 ± 5.4	8.9 ± 5.5	8.0 ± 4.7	8.2 ± 3.8			
			1–18	1–18	1–18	1–18	1–14			
		end	53.0 ± 41.2	73.5 ± 46.9	57.7 ± 56.3	140.5 ± 83.3	27.9 ± 20.0			
			5–168	10–143	7–206	32–297	2–87			
3	7	beg.	4.4 ± 4.5	5.0 ± 4.3	4.4 ± 3.8	4.2 ± 3.3				
			1–18	1–16	1–12	1–10				
		end	32.5 ± 26.9	21.9 ± 19.9	61.2 ± 57.7	50.6 ± 46.6				
			7–93	1–68	5–199	7–159				
4	8	beg.	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0
			1	1	1	1	1	1	1	1
		end	9.7 ± 7.5	19.8 ± 15.0	9.6 ± 3.7	6.9 ± 7.8	59.3 ± 28.4	9.7 ± 9.7	8.3 ± 4.5	9.8 ± 6.8
			1–32	5–57	5–18	3–34	21–119	2–45	2–16	4–23
5	7	beg.	1.2 ± 0.8	1.1 ± 0.4	1.1 ± 0.4	1.2 ± 0.4	1.1 ± 0.4	1.1 ± 0.4		
			1–4	1–2	1–2	1–2	1–2	1–2		
		end	7.6 ± 6.3	24.5 ± 13.3	16.1 ± 9.6	45.4 ± 33.1	35.7 ± 18.1	16.4 ± 8.8		
			2–18	4–45	1–37	13–142	3–63	8–38		
6	7	beg.	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0	
			1	1	1	1	1	1	1	
		end	50.1 ± 25.8	34.4 ± 20.1	15.1 ± 8.9	41.1 ± 33.4	39.0 ± 24.6	23.8 ± 15.4	30.7 ± 12.2	
			18–101	13–80	3–41	4–104	13–115	2–72	12–57	

* For each tank, the first line is the average colony size ($\bar{X}$ ± SD), the second line is size ranges (zooids).

Table III. Morphometric qualities for *Botryllus schlosseri* colonies in the six sets of inland culture experiments. Numbers in parentheses correspond to the number of colonies.

Experimental Set	Criteria tested	Tank No.							
		1	2	3	4	5	6	7	8
1	% survival	100 (10)	80 (10)	100 (10)					
	Health index*	2.0	1.8	2.0					
	% in reproduction **	0;0	0;0	0;0					
2	% survival	75 (20)	100 (20)	95 (20)	65 (20)***	100 (20)			
	Health index	3.1	2.7	2.6	2.6	3.0			
	% in reproduction	25; 69	75; 15	84; 11	46; 46	65; 15			
3	% survival	100 (20)	95 (20)	100 (20)	80 (20)				
	Health index	2.4	2.6	2.3	3.1				
	% in reproduction	15; 30	10;47	30; 30	20; 5				
4	% survival	94 (16)	100 (16)	100 (16)	100 (16)	87 (16)	100 (16)	94 (16)	69 (16)
	health index	2.1	2.4	2.4	3.4	2.6	2.3	2.4	2.0
	% in reproduction	6;0	19; 12	56; 12	0; 6	50; 38	31; 38	0; 0	0; 0
5	% survival	100 (14)	93 (14)	100 (14)	100 (14)	100 (14)	100 (14)		
	Health index	1.8	2.6	2.6	3.4	3.0	2.4		
	% in reproduction	0; 21	23; 54	69; 0	36; 14	36; 50	36; 14		
6	% survival	70 (20)	95 (20)	100 (20)	100 (20)	95 (20)	100 (20)	100 (20)	
	Health index	3.6	2.1	1.3	2.5	3.0	2.4	3.2	
	% in reproduction	0; 6	37; 42	0; 0	15; 50	16; 53	20; 60	10; 35	

* Health index = mean values for all colonies in the tank: 1 = bad, 2 = fair, 3 = good, 4 = very good (see materials and methods).
** for both types of sexual reproductions: % hermaphrodites; % males only
*** accidentally damaged

dead animal in each one of 26 aquaria) and was not related to the type of food or to the average growth rate (*table III*). For example, in Exp. 6, a very low survivorship (70 %) was recorded in aquarium no. 1 with the fastest growth rate (*tables II, III*). The same holds for the general health index. High or low growth rates or survivorship was not reflected by corresponding high or low 'health indices' (*tables II, III*). In most aquaria,

the average health index was around 3, equivalent of a 'good' state of health. Most colonies in all experiments had a clean, semi-transparent tunic free of algae or sedentary micro-organisms, had ramified active blood systems, active moving ampullae with fast circulating blood cells in the periphery of the colonies, contained relatively few numbers of pigment cells and were characterized by synchronized blastogenesis cycles.

3.2. Reproductive activity

High variations in reproductive activities between tanks was also a characteristic of the results obtained in the 6 experiments (*table III*). Reproduction was not directly related to fast growth rates (*table II*). For example, in the fastest growing group of animals in Exp. 6 (tank 1, *tables II, III*), almost no reproductive activity was recorded at the end of the experiment while 88 % of the colonies in the fastest growing group of Exp. 4 were in reproduction (hermaphrodites and male colonies; tank 5, *tables II, III*), the highest number in that experiment. No reproduction was recorded in only 6 of the total 33 aquaria used, (including the 3 tanks of Exp. 1, *table III*). In 3 tanks (a single aquarium in each of Exp. 4–6) only male gonads were developed in some of the colonies. No correlation could be found between these results and the type of food provided (*table I*).

We have also looked for the growth vs. reproductive activities within each single tank. The analyses were performed on the data collected during the last weeks of experiments 4–6, where colonies were put under different diet regimens immediately after settlement. We recorded reproduction in 18 out of the 21 aquaria. In 16 aquaria (except for tank no. 5, Exp. 5 and tank no. 1, Exp. 6), the animals that were reproductive also grew on average faster than those that were not. This difference was statistically significant (*t*-test, $P < 0.05$) in 7 aquaria (43.8 %; Exp. 4, tanks 2, 3, 6; Exp. 6, tanks 1, 2; Exp. 6, tanks 4, 6).

3.3. Inbred line

Colony no. M 1965, heterozygotic for the fusibility locus (Fu/HC; 39), was born in the laboratory in February 1991, grew fast and was reproductive within 13 months. We named its two fusibility alleles δ and γ, since they differ from alleles α and β designated earlier for Monterey *Botryllus* colonies [30], when the colony allorecognition assay [26] against an αβ colony was done. Colony M1965 was subcloned to > 20 ramets which were put into two separate aquaria. Within the next 3 years, colony M1965 produced 561 fast growing offsprings under laboratory conditions (*figure 2*). F1 colony S168 (born May 1992) was subcloned to several ramets and entered into intensive sexual reproduction within 6 months. During the next 5 months, we collected 243 offspring, of which colony S68 (born March 1994; γγ on the fusibility locus) grew fast, was subcloned and within one month, started to release offspring of the F3 generation from self crossed ramets (a total of 164 offsprings within the next 15 months, all γγ on Fu/HC). Colony S1 of the F3 generation began sex-

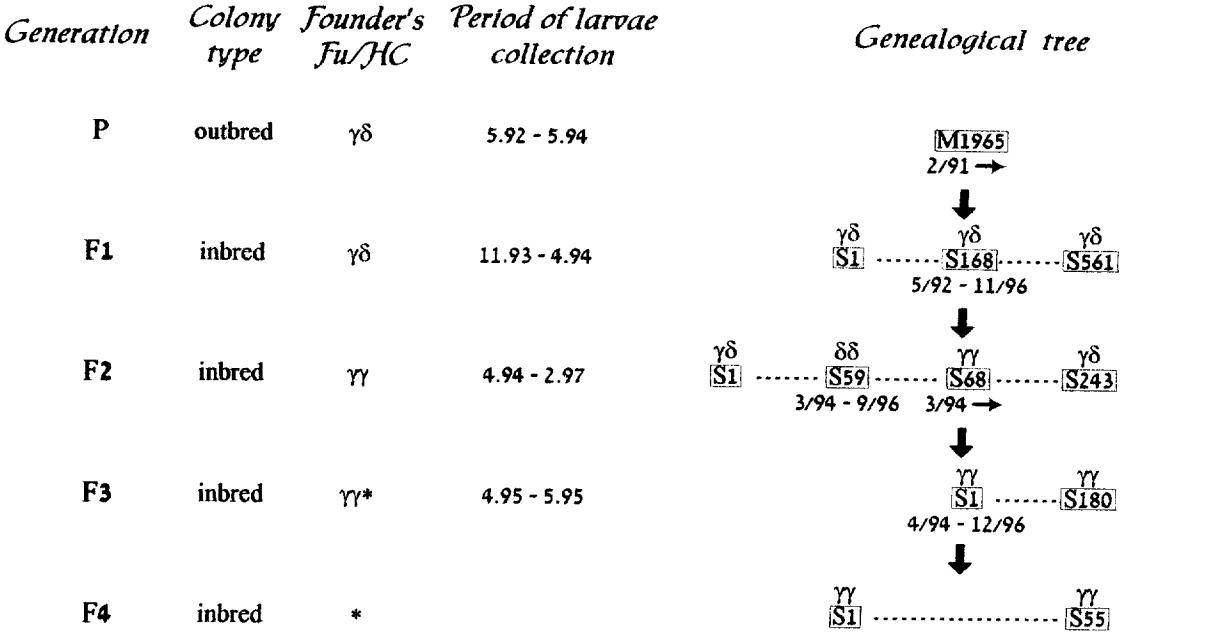

Figure 2. The pedigree and genealogical tree for 5 successive generations of *Botryllus schlosseri*. Only colonies that successfully settled, metamorphosed and developed are included. Colonies of generations F1 to F4 are designated by running numbers according to their date of birth. The preface letter *S* stands for self–crossing. Founder colonies are further marked for days of birth and death (except for those that are still alive). Asterisks denote that all colonies in the specific generation have the same Fu/HC allele.

ual reproduction one year after settlement and released 55 successful offsprings within two months (F4 generation, all γγ on Fu/HC; *figure 2*). In order to assess Fu/HC allelic combinations, colony allorecognition assays between colonies of a specific generation were done as described [25]. Colony S68 rejected S59 and therefore both are homozygotes. All F3 and F4 offsprings fuse with the others.

4. DISCUSSION

Improved inland maintenance and culturing methods of animals are crucial for the development of inbred lines of marine invertebrates available for a variety of commercial and scientific purposes. The culture conditions should not only keep the animals in good physiological status (such as fast growth), but should also support sexual reproduction, high survivorship of young animals and long-term maintenance of the animals under laboratory conditions. This is especially true for the growing of inbred lines of marine invertebrates known to be characterized by high variability in their life history patterns [16]. Success in cultivation of any specific species of marine invertebrate depends on the ability to develop critical inland protocols circumventing the physical conditions in nature.

Past studies on *Botryllus schlosseri* [3, 4, 18, 27, 30, 33] showed that colonies can successfully be maintained in inland culture. Although the construction of inland conditions to obtain *Botryllus* colonies with various life cycle characteristics to satisfy different experimental needs has already been developed [3, 27], there is no available long-term inbred line. The previously described short inbred line from *B. schlosseri* [30] included only two successive, self-crossed generations. It is therefore a proven achievement that our maintenance protocols has enabled us to culture one line of self-fertilized *Botryllus* colonies for more than 7 years, and we are now raising the fourth generation of self-crossed animals in this pedigree. This is the first such long-lived inbred line of *B. schlosseri.*

The results of the present study show that a mixture (best ratio is 1:1) of two types of diets is superior to any monotype diet, especially when the total amount of food supplied in both cases was the same. Therefore, a satisfactory feeding protocol for *Botryllus schlosseri* under inland cultures is based, on a multicomponent nutrition type rather than on increased food quantity. A previous study had already documented that increasing the concentration of algal cells in the medium was accompanied by a slow growth rate and poor appearance of the colonies [18].

Botryllus colonies under our culturing conditions showed high survivorship. Many of them are long lived. The founder colony for the inbred line (No. M1965, *figure 2*) which was born in February 1991 is still alive, more than 7 years later. This is probably the longest recorded life span in this group of organisms which are known to be seasonally short lived [2, 3, 5, 6, 10, 19, 29]. Moreover, sexual reproduction was frequently recorded within the first two months after tadpole larvae settlement and metamorphosis, and reproductive colonies were the same size or significantly larger than sterile colonies. This indicates that sterility was not the result of poor culture conditions but the physiological outcome for colonies that either do not fit the inland conditions or animals that suffer from genetic disorders resulting from fertilization between genetically related colonies [34]. Additionally, most colonies in our cultures showed a high 'state of health' condition. Therefore, these long-lived, healthy animals are suitable for a variety of scientific needs including the development of inbred lines for immunological assays, developmental biology experiments and more. These animals have probably escaped the inbreeding depression problem [34].

As in previous laboratory results [3, 29] or field studies [5, 6, 10, 40], *B. schlosseri* colonies were characterized by high variability in every studied aspect of their life history pattern. Colonies from the same hatch of a specific mother colony, under the same culturing conditions and even within the same aquarium, showed differences in growth rates which exceeded one order of magnitude or showed high, intensive reproductive states versus sterility. This natural characteristic of *B. schlosseri* is one of the major drawbacks for continuous maintenance of high numbers of colonies, since many of the inland raised animals do not have the specific requirement needed for their culture. The colonies cultured with the methodology described here, those from the inbred line and those developed in the six sets of experiments, have already been used for a variety of scientific studies such as the nature of alloimmune responses. We are, therefore, at the beginning of a long-term study to develop several lines of highly inbred *B. schlosseri*, and such a goal is now feasible.

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