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AN INDEX OF AGE WHEN BIRTHDATE IS UNKNOWN IN *APLYSIA CALIFORNICA*: SHELL SIZE AND GROWTH IN LONG-TERM MARICULTURED ANIMALS

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Abstract

The purpose of this study was to determine the age of postmetamorphic *Aplysia* when the birthdate was not known. The study is based upon the long-term growth and maintenance of *Aplysia californica* kept in artificial sea water up to 235 days. Body and organ weights and organ size were measured. The most reliable measure of growth and age was the size of the internalized shell.

The rate of shell growth was determined, and on the average it was 0.23 mm/day throughout post-metamorphic life. A means of determining age using shell size is given. The results reported here are used to determine the age of *Aplysia* in which age-dependent changes in behavior and in anatomical and physiological properties of single nerve cells have been found.

Introduction

Aplysia californica is a marine gastropod mollusc with a life span of approximately one year (Eales, 1921; Kandel, 1976; Audesirk, 1979). Laboratory studies revealed that *Aplysia* pass through both an embryonic and a larval stage before developing into the adult form: after oviposition, fertilized eggs undergo embryonic development of 8–10 days and then, as is typical for marine gastropods, pass through a veliger larval stage for 30–34 days; metamorphosis lasts for 2 to 3 days (Kriegstein *et al.*, 1974; Kriegstein, 1977; Switzer-Dunlap and Hadfield, 1977). Immediately after metamorphosis occurs, a miniaturized version of the larger and older post-metamorphic animal appears. *Aplysia* can be cultured in the laboratory from oviposited eggs to sexually mature animals in natural sea water (Kriegstein *et al.*, 1974; Switzer-Dunlap and Hadfield, 1977), but as yet not in artificial sea water (Kriegstein, personal communication; Peretz, unpublished observations). At present there is no reliable means of determining age of *Aplysia* other than body weight without knowing the birthdate. Body weight is subject to considerable variation (Audesirk, 1979). Most studies on the *Aplysia* nervous system, which contains identifiable neurons, have been done on post-metamorphic animals (for review see Kandel, 1976) of unknown age.

Peretz and Lukowiak (1975) found physiological and behavioral differences between young and sexually mature *Aplysia*, both of which were post-metamorphic groups. Their results prompted further studies of age-dependent neurophysiological, neuroanatomical, and behavioral changes in *Aplysia*. Recent studies showed that the *Aplysia* nervous system with its identifiable neurons is a useful preparation to study the life history of single neurons (Papka *et al.*, 1981; Rattan and Peretz, 1981; Peretz *et al.*, 1982). Characteristics of aging in *Aplysia* neurons show a

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striking resemblance to those in mammalian neurons (Papka *et al.*, 1981). It was necessary to determine the age of post-metamorphic animals whose birthdate was not known with reliability greater than that obtained using body weight. The literature dealing with molluscan growth and allometry is extensive with respect to animals studied in the field (Wilbur and Owen, 1964; see also Comfort, 1957; Huxley, 1972). Laboratory studies of the growth of gastropod molluscs are less extensive (Carefoot, 1967; Kriegstein *et al.*, 1974; Kempf and Willows, 1977; Kriegstein, 1977; Switzer-Dunlap and Hadfield, 1977). The findings reported here provide a means of determining age of post-metamorphic *Aplysia*.

Materials and Methods

Post-metamorphic *Aplysia californica* obtained from Pacific Biomarine, Inc. (Venice, California) ranged in weight from 0.1 g to 1100 g. In this study, begun in January 1979, body weight and organ measurements were made in 378 animals. Upon arrival, animals were weighed and tagged for identification and then placed in holding tanks. Plastic tags which were numbered and color-coded were attached to the posterior portion of one of the parapodia by surgical thread. A loop of thread was used so that it did not interfere with growth of the parapodium; thread tied too tightly to the parapodium eventually cut through the tissue and fell off. Very young animals, kept in perforated refrigerator containers in the holding tanks, were tagged after attaining a body weight of 50 g. Each animal was weighed once a week. Damp weight of animals was subject to less variation on repeated measures than attempting to determine the "maximum stretched" length by anesthetizing animals with an injection of $MgCl_2$. It was felt that the possible cumulative effects of repeated anesthetization with $MgCl_2$ on subsequent neurophysiological and behavioral experiments should be avoided. When an animal was sacrificed, the reproductive tract, radula, gill, and shell were weighed. Maximum shell diameter was also measured.

All animals in this study were well past metamorphosis. In post-metamorphic animals, the tissue constituting the mantle flap covers the shell (see Kriegstein, 1977); this structure strengthened by the shell is located in the pallial cavity overlaying and protecting the gill. The shell was removed by cutting away the mantle tissue and gently removing it from its attachment at the base of the mantle. The shell contains bands analogous to the rings seen on shells of bivalve molluscs. The distinctness of the bands was highly variable from animal to animal and thus could not be counted nor used as an index of growth. Also, growth rings on the shells of certain species of bivalves are affected by environmental conditions and thus are not solely expressions of growth and age (Jones, 1981); this may hold also for the *Aplysia* shell. The method used to measure the maximum shell diameter was similar to that described by Wilbur and Owen (1964) for bivalves. The diameter is the perpendicular distance from the umbo to the line drawn tangent to the point of maximum curvature (inset Fig. 5).

Animals are kept in artificial sea water (Instant Ocean, Eastlake, Ohio). The 450 gallon recirculating system consists of three 50 and two 10 gallon holding tanks, two reservoirs of 145 gallons each and a filter; metal-free pumps (March Pumps, Glenview, Ill.) are used to circulate the sea water. The temperature is held at 16 ± 1°C throughout the year by passing it through plastic tubing submerged in a 55 gallon container of chilled tap water. The specific gravity is maintained at 1.023 to 1.024 by adding tap water to the reservoir when necessary. The sea water is passed through a filter composed of a bottom layer of activated charcoal, 5 inches

thick, which removes finely suspended material; above it a layer of crushed oyster shell, 6 inches thick, which is used to buffer the sea water at pH 7.8 ± 0.1; and above that a porous polyurethane mat prevents passage of larger pieces of fecal matter and detritus. The system is cleaned once a week.

Animals are fed five times per week: the smallest animals, up to 10 g are fed fresh *Plocamium* and those 10 g and over are fed dried red laver, *Porphyra*, (Vega Trading Co., New York) twice per week and fresh *Lactuca sativa longifolia*, romaine lettuce, three times per week.

Results

The objective of this study was to find a convenient and reliable index of growth in post-metamorphic animals and consequently to determine their age under laboratory conditions—that is, in artificial sea water, invariant temperature, fixed photoperiod of 12:12, and dried seaweed and soil-grown lettuce. As will be seen below, rate of body weight increase was found to be similar to that of animals raised under conditions more closely resembling those in the field. The rate of increase was dependent on size rendering it an unreliable index of age. Also, considerable weight change results from egg laying.

General observations

Animals that consistently gained weight, regularly ambulated in the holding tanks, and laid eggs after attaining sexual maturity survived the longest. Of the 236 animals whose weights were recorded for more than two weeks, 84 percent gained weight.

Loss of weight for three consecutive weeks usually signalled impending death. Loss of weight also followed egg laying (Fig. 1). In sexually mature animals (100–250 g) the weight was regained. This is consistent with the observation of Audesirk (1979). In contrast, in old animals (500 g and greater) weight loss was not regained (Fig. 1). Regaining of weight after egg laying appears to be age-dependent.

Of the 378 animals studied, 1.6 percent died within two weeks of their arrival; the mortality rate of the animals kept beyond two weeks was only 19 percent. Deaths were not correlated with a particular season. Animals received in winter survived well into spring; those received in the summer survived beyond winter. This observation suggests that death of *Aplysia* is not a seasonal event, and that their survival is dependent upon internal factors rather than environmental factors.

Growth as measured by body weight

Animals were weighed once a week, and growth rates as expressed by increases in body weight were measured (Fig. 2A). Young animals (juveniles) whose average weight upon arrival was 1.7 g were cultured in the holding tanks for 105 days at which time they had grown to an average weight of 170 g. Growth follows a sigmoid-like curve. As is typical for growth curves, Figure 2A shows an initial "lag" phase. Body weight increases approximately 0.28 g/day. During "log" phase body weight doubles every 14 days from the 28th day, the rate increases to 2.1 g/day. Body weight doubles every 7 to 10 days up to 100 g and then approximately once every 25 days.

When larger animals (mature) are cultured and body weight measured over a comparable period of time, the growth rate is different than that in Figure 2 (curve A). Figure 2 (curve B) shows that a lag phase is absent and the growth is

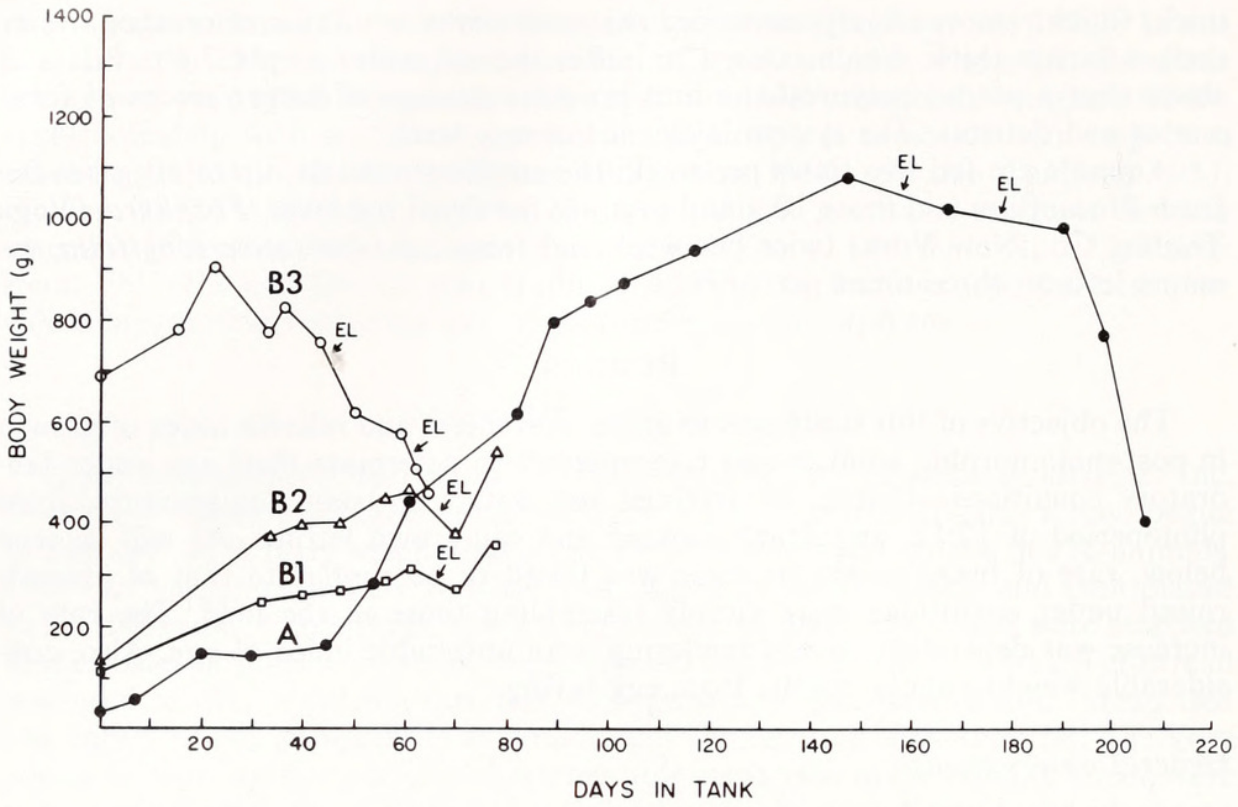

FIGURE 1. An animal cultured from an initial weight of 12 g, arrival date 26 January, 1979, to a maximum weight of 1090 g (curve A). Egg laying (EL) occurred 160 days after arrival. Note that animal gained 150 g within one week and lost 400 g within one week. The variability in weight changes seen here is typical for animals in this study. Three animals from the same shipment showing the effect of egglaying (EL) on body weight (curve B1–3). The upper curve is from an old animal who did not recover from weight loss. The two lower curves are of mature animals approximately 70 days younger than the old animal, which show recovery of body weight. Spontaneous egg laying occurred in the holding tanks throughout the calendar year rather than during a specific egg laying season.

more linear. The growth rate of 3.6 g/day is greater than that for younger animals. Animals 500 g and larger have a growth rate of 7.6 g/day. Table I shows that growth rates vary considerably from weight group to weight group, with the coefficient of variation ranging from 13 to 57 percent and averaging 30 percent. The rate of increase of body weight appears dependent upon the age of the animal. These results in addition to those in Figure 1 show that body weight is not a reliable index of age.

With the kind offer from Dr. J. Vallee (Pacific Biomarine, Inc.) of a growth curve of animals cultured from eggs, we were able to compare growth in the holding tanks with animals grown in natural sea water (Fig. 2B). Animals grown from eggs were cultured at 18 to 20°C and fed fresh seaweed *ad libitum*. The growth rate during "lag" phase appears to be 0.25 g/day. The growth rate during "log" phase is approximately 3 g/day which is comparable to that of growth in artificial sea water. In another study in which animals were raised from oviposited eggs in natural sea water at 22°C and fed regularly, the growth rate of young animals nearing sexual maturity was approximately 6 g/day (Kriegstein *et al.*, 1974; Kriegstein, personal communication). The difference between Kriegstein's results and ours probably is due to the difference in ambient temperature, 22°C *vs* 16°C. The difference between Vallee's results and those of Kriegstein could be explained by the difference in feeding regimen and possibly the difference in temperature.

During 2.5 years of measuring body weights seasonal variations in the growth

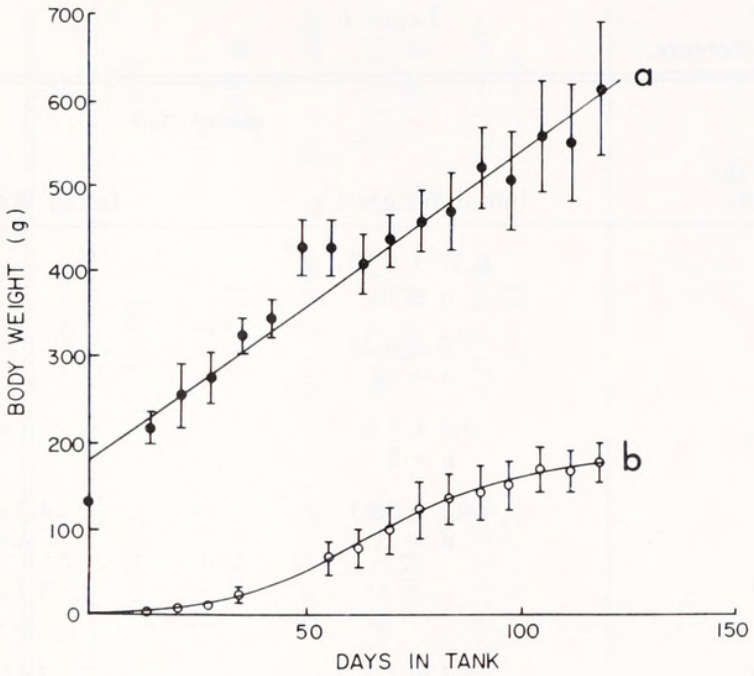

FIGURE 2(A). Growth curve of animals whose average initial weight was 1.7 g (curve A). The average body weight of the group (n = 10) is plotted against days cultured in tanks. The animals were raised from young to sexually mature animals. The equation which best relates body weight (w) to time (t) in the holding tanks is: $w = 3.4 - 0.8t + 0.5t^2 - 2.4 \times 10^{-4}t^3$. This equation only holds for animals up to 200 g. Growth curve of animals whose average initial weight was 132 g (curve B). The average body weight of the group (n = 8) is plotted against days cultured in tanks. The animals were raised from sexually mature to old animals. The growth rate is comparable to those animals of curve A and Figure 2B whose weights exceed 100 g. The equation which best relates body weight (w) to time (t) in the holding tanks for mature animals is: $w = 182 + 3.6t$. This equation only holds for animals above 100 g.

rate have not been observed. It is possible that seasonal effects disappear when animals acclimate to conditions in the holding tanks after two weeks.

Organ weight in relation to body weight

An allometric study was carried out between reproductive tract, radula, gill, and shell weights and body weight (Fig. 3). Body weight *vs* organ weight was plotted logarithmically, because allometric relationships obey the power law, *i.e.*, $y = ax^b$ (von Bertalanffy, 1960; Jones, 1981). (The equations for the best fit line are given in Figure 4.) Minimal change of shell and radula weights was observed up to a body weight of 50 g. The organ weights of animals above 50 g increase with considerable scatter. At a given body weight the coefficient of variation is on the average 49 percent for shell weight and 26 percent for radula weight. The relationship of gill and reproductive tract weights to body weight also displays considerable scatter. At a given body weight the average coefficient of variation of gill weight is 38 percent and 51 percent for reproductive tract weight. The use of any of the four organ weights would result in a less reliable estimate of age than using body weight alone.

Shell size in relation to body weight

A more consistent relationship was found between maximum shell diameter and body weight, plotted logarithmically (Fig. 4). The shell diameter increases with body weight throughout the post-metamorphic life of *Aplysia*. At a given body

TABLE I

Rate of body weight increase.

Weight range (g) during growth	grams/day Initial Weight 1 g	Initial Weight 120 g
1–10	0.57 ± 0.33 n = 15	—
10–50	1.0 ± 0.23 n = 12	—
50–120	3.3 ± 1.6 n = 9	—
120–500	4.2 ± 0.53 n = 11	4.3 ± 0.58 n = 4
120–1200	—	5.7 ± 1.5 n = 9
500–1200	5.3 ± 1.2 n = 4	7.4 ± 2.8 n = 9

weight shell diameters of recent arrivals, sacrificed within two weeks, and animals in the holding tanks for longer periods, up to 235 days, were the same. The coefficient of variation for the diameter at a given body weight does not vary more than 8 percent. Non-linear regression analysis of the data yielded a best fit curve given by the equation, $Y = 5.1 \times W^{0.33}$ (n = 347); where Y is the shell diameter and W is the body weight; the correlation coefficient is 0.99. The form of the

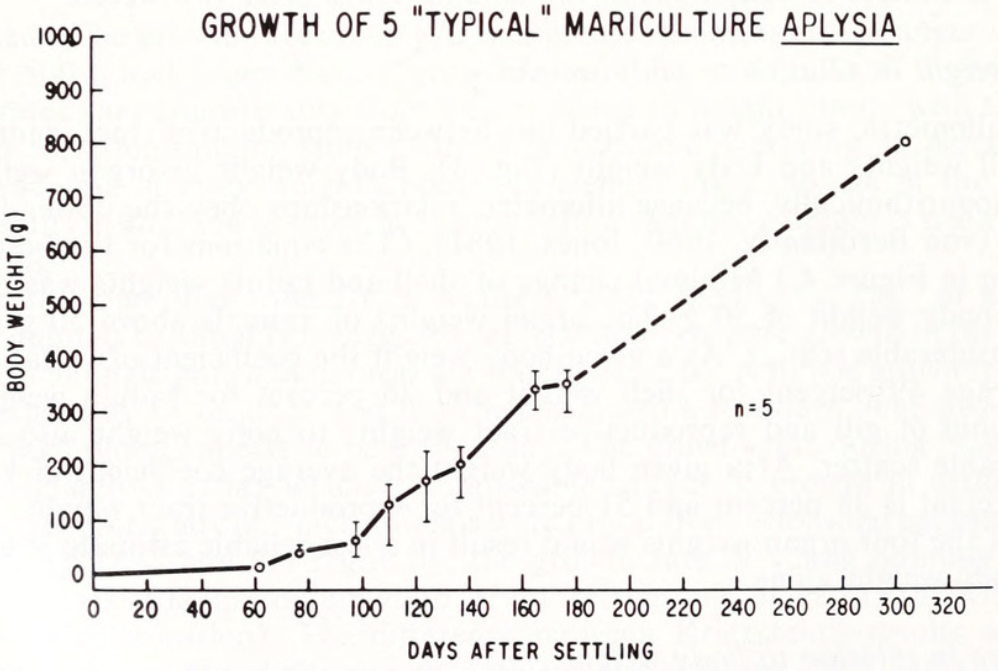

FIGURE 2(B). Growth curve of animals cultured from oviposited eggs in natural seawater at 20°C and fed fresh seaweed. Animals at 60 days after settling are equivalent to animals in (A) at zero time in tanks. Note the initial lag phase and subsequent log phase. The growth rates in A and B are comparable. (Modified from curve supplied by Dr. J. Vallee.)

TABLE II

Rate of shell growth (mm/day).

	1*	2*	3†	4‡	5*	6*	7*
	[DS]	[SS]	[DS]	[DS]	[SS]	[DS]	[DS]
Initial SD—mm	4.6 ± 0.4 (0.9 g)	4.6 ± 0.4 (0.9 g)	4.6 ± 0.4 (0.9 g)	25.1 ± 2.2 (100 g)	25.1 ± 2.2 (100 g)	25.1 ± 2.2 (100 g)	41.2 ± 3 (500 g)
Growth Interval	131 ± 32 days	100 ± 53 days	79 ± 26 days	51 ± 29 days	85 ± 27 days	90 ± 29 days	30 ± 13 days
Final SD—mm	30.1 ± 2.4 (220 g)	35.6 ± 4.7 (385 g)	15.8 ± 2.4 (30 g)	30.5 ± 2.4 (250 g)	41.2 ± 3.0 (500 g)	41.2 ± 3.0 (600 g)	53.3 ± 3.1 (1200 g)
Rate mm/day ± SE	0.24 ± 0.02 n = 10	0.28 ± 0.04 n = 8	0.13 ± 0.03 n = 7	0.14 ± 0.01 n = 9	0.21 ± 0.02 n = 17	0.21 ± 0.03 n = 11	0.21 ± 0.05 n = 8

Weighted average of columns 1, 2, 5, 6, and 7: 0.23 ± 0.02 mm/day, n = 54

* No significant difference between columns 1, 2, 5, 6, and 7, $P > 0.1$ (Mann-Whitney Test).
† Significant difference between columns 1 and 3; $P < 0.05$ (Mann-Whitney Test).
‡ Significant difference between columns 4 and 5; $P < 0.05$ (Mann-Whitney Test).
[DS] Animals from different groups.
[SS] Animals from same group.
Average body weight in parentheses.

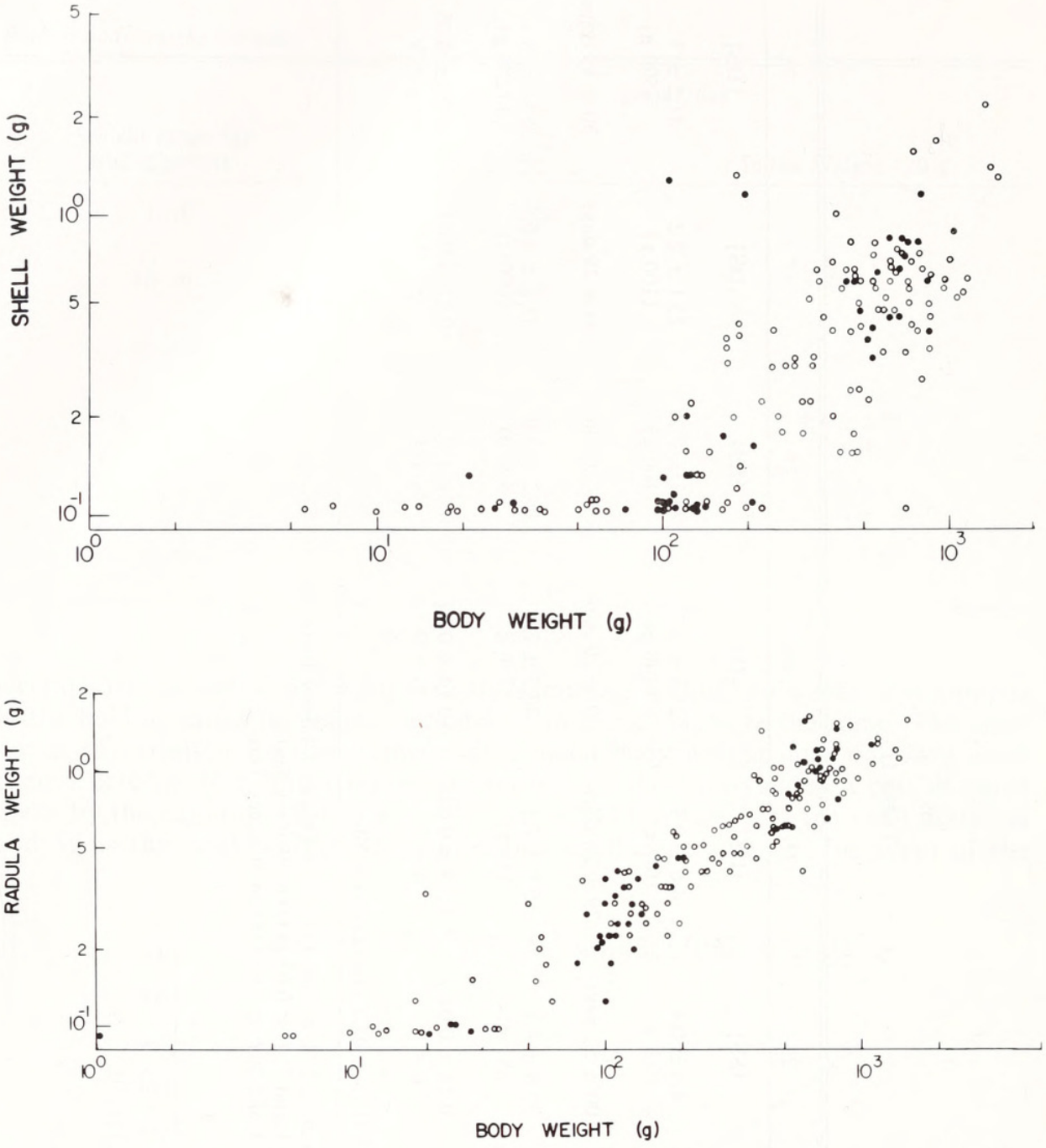

FIGURE 3(A), (B), (C), (D). Relationship between organ weights and body weights plotted logarithmically. Closed circles (●) indicate measurements taken from newly arrived animals, and open circles (○) are those from animals cultured for at least 30 days. Note that there is no difference between newly arrived and cultured animals. The equations for the best fit line for the allometric relationships is given below:

(A) Shell wt *vs* BW $Y = 3 \times 10^{-3}X^{0.78}$

(B) Radula wt *vs* BW $Y = 8 \times 10^{-3}X^{0.74}$

(C) Gill wt *vs* BW $Y = 9 \times 10^{-3}X^{0.95}$

(D) Reproductive tract wt *vs* BW $Y = 4 \times 10^{-3}X^{0.99}$

equation is the same as that for relating shell diameter to body weight in bivalve molluscs (Wilbur and Owen, 1964). Also Huxley (1972) has shown that organ size or length of a body part varies with the cube root of body weight (see also von Bertalanffy, 1960).

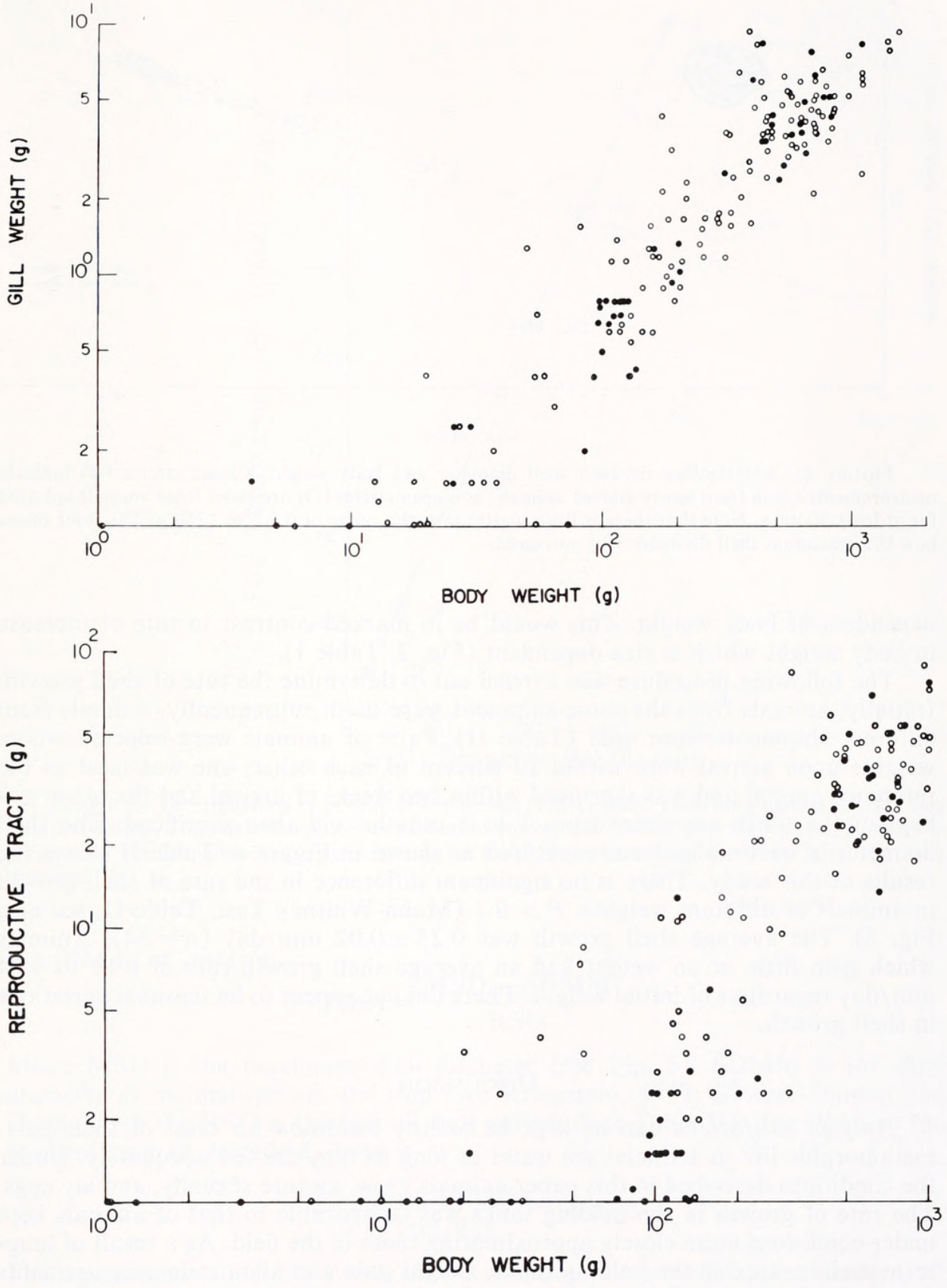

FIGURE 3. (Continued)

Rate of shell growth

We sought to determine the rate of shell growth and investigated the possibility that the rate of shell growth is the same in small and large animals, that is, in-

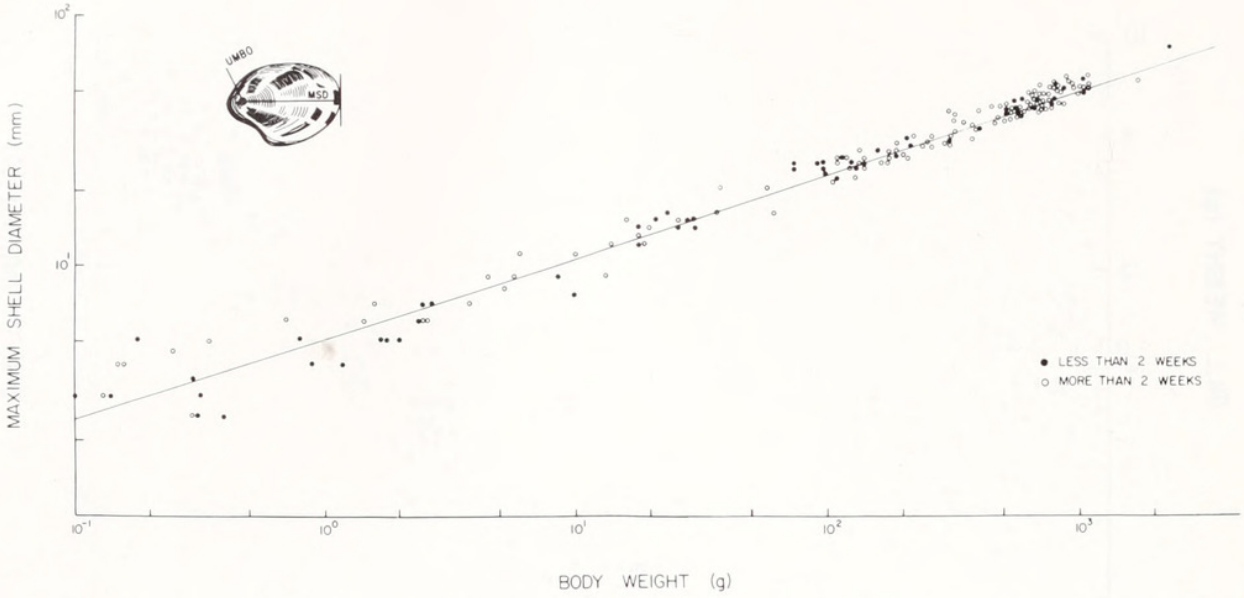

FIGURE 4. Relationship between shell diameter and body weight. Closed circles (●) indicate measurements taken from newly arrived animals, and open circles (○) are those from animals cultured for at least 30 days. Note that there is little scatter over the range of 0.5 g to 1250 g. The inset shows how the maximum shell diameter was measured.

dependent of body weight. This would be in marked contrast to rate of increase in body weight which is size dependent (Fig. 2, Table I).

The following procedure was carried out to determine the rate of shell growth. Initially, animals from the same shipment were used; subsequently, animals from different shipments were used (Table II). Pairs of animals were selected whose weights upon arrival were within 10 percent of each other; one was used as the reference animal and was sacrificed within two weeks of arrival and the other was kept in the tanks anywhere from 1 to 5 months and then sacrificed. The shell diameter of each animal was measured as shown in Figure 4. Table II shows the results of this study. There is no significant difference in the rate of shell growth in animals of different weights, $P > 0.1$ (Mann-Whitney Test, Table II; see also Fig. 5). The average shell growth was 0.23 ± 0.02 mm/day ($n = 54$). Animals which gain little or no weight had an average shell growth rate of 0.13 to 0.14 mm/day regardless of initial weight. There did not appear to be seasonal variations in shell growth.

DISCUSSION

Aplysia californica can be kept in healthy condition for most of their post-metamorphic life in artificial sea water as long as they are fed adequately. Under the conditions described in this paper animals grow, mature sexually, and lay eggs. The rate of growth in the holding tanks was comparable to that of animals kept under conditions more closely approximating those in the field. As a result of long-term maintenance in the holding tanks, weight gain and allometric measurements of organs were made to find a reliable index of age. Of the measurements made, the most reliable found was the rate of shell growth. The results are consistent with previous studies (Tessier, 1960; von Bertalanffy, 1960; Wilbur and Owen, 1964; Huxley, 1972, Audesrik, 1979).

Using the rate of shell growth obtained from matched pairs in Table II and Figure 5, we developed the following equation to determine the postmetamorphic

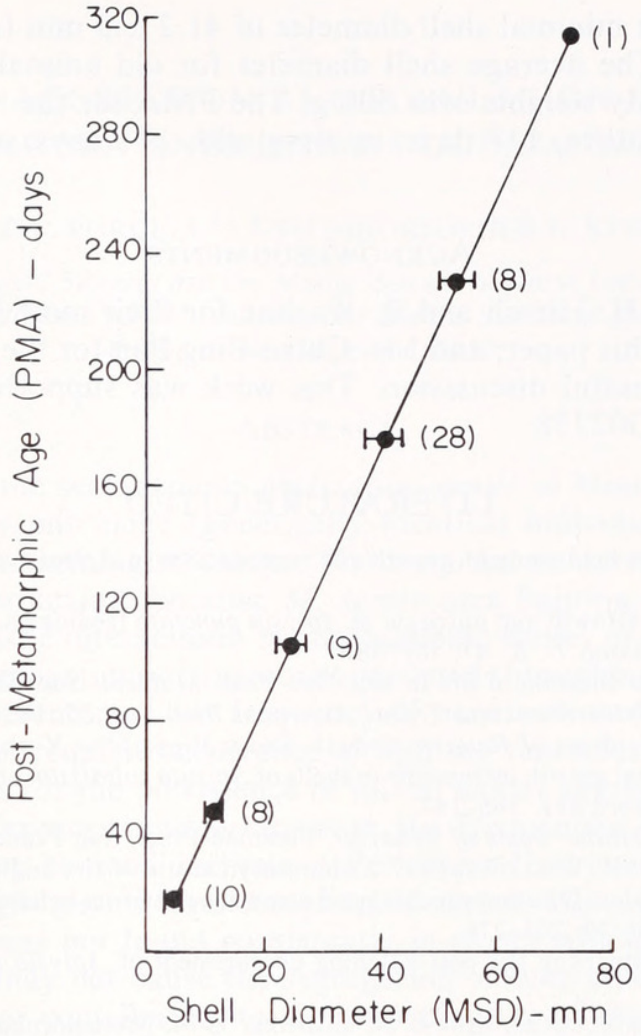

FIGURE 5. Determination of postmetamorphic age (PMA) based upon the equation given in text (see Discussion). The relationship in the equation is entirely independent of body weight. Numbers in parentheses are the number of determinations for each point plotted; standard error given for each shell size.

age (PMA) of *Aplysia*:

$$\text{PMA} = \frac{\text{MSD} - \text{SDMM}}{\text{RSG}};$$

where MSD is the maximum shell diameter (see Fig. 5); SDMM is the shell diameter at metamorphosis, 0.4 mm (see Kriegstein, 1977; Switzer-Dunlap and Hadfield, 1977); RSG is the rate of shell growth (See Table II). The PMA of the smallest animals described above is

$$\frac{4.6 \text{ mm} - 0.4 \text{ mm}}{0.23 \text{ mm/day}} = 18 \text{ days}$$

This age compares favorably with that of animals whose birthdate was known, 17 days after metamorphosis, as described by Kriegstein (1977, Table I).

It is now possible to determine the PMA of the age groups studied previously (Papka *et al.*, 1981; Rattan and Peretz, 1981; Peretz *et al.*, 1982), using the equation above or Figure 5. Young animals have an average shell diameter of 10.4 ± 3.5 mm (n = 24), with body weights from 2 to 20 g. Mature animals have an average shell diameter of 27.4 ± 4.4 mm (n = 92), with body weights from 100 to 250 g.

Old animals have a minimal shell diameter of 41.2 ± 3 mm (n = 21), with a body weight of 500 g. The average shell diameter for old animals was 47.6 ± 4 mm (n = 106), with body weights over 500 g. The PMA for the three age groups are: young, 43 days; mature, 117 days; minimal old, 177 days; and average old, 205 days.

Acknowledgments

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