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Axenic Ovarial Explants from the Marine Nematode *Deontostoma californicum* on Culture Media

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Abstract: The gonads of *Deontostoma californicum* were isolated from the organismal environment by dissection. In an attempt to approximate the *in vivo* condition and to maintain *in vitro* architecture and function ten media recommended for the culture of insect cells or organs were tested. The media were prepared in two series: one according to published recommendations and the second substituting filtered seawater for the inorganic salts and distilled water. Media were prepared as both liquid and gel (agar) with and without antibiotics. Whole animals and eggs were placed on the same culture media series as the ovarial explants. Ovarial explant reaction was divided into those which supported the entire gonad and those which supported the ovary or ova-containing oviduct. Seawater preparations of Samia and Grace culture media were outstanding for support of the entire explant. Eggs underwent their greatest development in seawater without antibiotics. Prolonged survival of whole animals took place only in those media (A-1, C-G, 26c, 199, Mosquito, and Media B) in which the inorganic salts and distilled water were replaced by filtered seawater. The results demonstrated that those conditions suitable for adults on culture media are not necessarily suitable for eggs, larvae, or tissue explants.

The successful rearing of nematodes, both parasitic and fresh-water forms, has been accomplished by many investigators (1, 2, 4, 5, 6, 8, 9). There are, however, no publications of the application of tissue culture techniques to explant organs or nematodes *in vitro*. The intent of this investigation was to isolate the gonad from the organismal environment and with the aid of tissue culture techniques approximate the *in vivo* condition in order to maintain *in vitro* the architecture and function of the tissues. Gonadal response was compared to fertile egg and whole animal reaction on the same media.

MATERIALS AND METHODS

The specimens of *Deontostoma californicum* Steiner and Albin, 1933 employed in this study were taken from holdfasts of *Egregria laevigata* and *Laminaria digitata* collected at Dillon Beach, California. After separation from the holdfasts, the population was segregated according to sex and stage: 25% juveniles, 8% gynandromorphs, 4% males, 64% females, and was stored in aerated seawater at 5 C.

Ten selected media were investigated and are representative of those recommended by Martignoni (7) for the maintenance, growth or differentiation of insect cells or organs cultured *in vitro*. The following media were tested: culture Medium B, CG Medium, Medium A-1, Medium for *Samia advena,* Synthetic Mixture 199, Medium K-6 for *Drosophila,* Physiological Solution for *Bombyx mori,* Medium for the mosquito phase of *Plasmodium relictum,* Medium 26c, Grace's insect T.C. Medium. The formulation of each media was according to Martignoni (7) except Grace's media (9).

Because *D. californicum* is a marine nematode, two basic media conditions were used for all explants, normal media and modified media with inorganic salts and distilled water replaced by sterile filtered seawater (SW). Seawater agar, as gel and liquid, was utilized as a control. Normal media and seawater media were prepared in four ways: liquid, liquid plus antibiotics, agar gel, and agar gel plus antibiotics. To maintain consistency in the gel properties of the stabilized media the agar concentration was adjusted to 0.37% in seawater and 0.50% in the distilled water media. All media were sterilized by filtration

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TABLE 1. The maintenance in days of cellularity in ovarial explants of *D. californicum*.

Media	Liquid				Liquid + A				Agar				Agar + A			
	Terminal cellularity		Terminal structure 21 days		Terminal cellularity		Terminal structure 21 days		Terminal cellularity		Terminal structure 21 days		Terminal cellularity		Terminal structure 21 days	
	Ovary	Ovdt.	Ovary	Ovdt.	Ovary	Ovdt.	Ovary	Ovdt.	Ovary	Ovdt.	Ovary	Ovdt.	Ovary	Ovdt.	Ovary	Ovdt.
Control (SW)[a]	4	11	CO[c]	CO	4	4	CO	CO	7	4	NS	NS	7	4	NS	NS
Bombyx (SW)	3	14	NS[d]	CO	7	14	NS	CO	3	3	NS	NS	3	7	NS	CO
Bombyx	14	14	NS	CO	14	14	CO	CO	3	7	CO	CO	3	3	CO	CO
Samia (SW)	14	21	CO	CO	14	14	CO	CO	14	14	CO	CO	14	14	CO	CO
Samia	7	7	CO	CO	7	7	NS	CO	3	3	NS	CO	3	3	NS	NS
A-1 (SW)	3	3	NS	NS	7	7	NS	NS	3	7	NS	NS	3	7	NS	CO
A-1	7	14	NS	CO	7	14	CO	CO	7	7	NS	NS	3	7	NS	CO
K-6 (SW)	14	14	CO	CO	7	14	NS	CO	14	14	CO	CO	14	14	CO	CO
K-6	7	14	CO	CO	14	14	CO	CO	14	7	CO	CO	14	14	CO	CO
C-G (SW)	3	3	NS	NS	7	7	NS	NS	14	7	CO	NS	3	7	NS	NS
C-G	3	7	NS	CO	3	7	NS	NS	3	3	NS	NS	3	3	NS	NS
Mosquito (SW)	3	7	NS	NS	3	3	NS	NS	3	3	NS	CO	3	7	NS	CO
Mosquito	3	7	NS	NS	3	3	NS	NS	3	3	NS	NS	3	3	NS	NS
26c (SW)	14	7	CO	CO	7	7	CO	CO	7	14	NS	NS	3	7	NS	CO
26c	3	3	NS	NS	3	3	NS	CO	3	3	NS	NS	3	3	NS	NS
Media B (SW)	3	7	NS	NS	3	7	NS	CO	7	3	CO	CO	7	3	CO	CO
Media B	3	7	NS	NS	7	14	NS	CO	7	14	NS	CO	3	14	NS	CO
199 (SW)	3	3	NS	NS	3	3	NS	NS	3	3	NS	NS	3	3	NS	NS
199	3	3	NS	NS	3	3	NS	NS	3	3	NS	NS	3	3	NS	NS
Grace (SW)[b]	60	60	CO	CO	21	14	CO	CO	30	30	CO	CO	21	14	CO	CO
Grace	3	3	NS	NS	3	3	NS	NS	3	3	CO	CO	3	3	NS	NS

[a] Inorganic salts and distilled H_2O replaced with sterile filtered seawater.
[b] Grace Seawater, agar-cellularity persisted for 8 months in two replicates. Grace Seawater, liquid + A-cellularity persisted for 8 months in one replicate.
[c] Cell outline.
[d] No structure visible.

through a millipore filter. The hot sterile agar solution was added to warm media, together with the respective antibiotic solution. Aliquant solutions of media were then poured into appropriate sterile receptacles, petri dishes (20 to 30 ml for whole animals) and embryo dishes (2 to 3 ml for explanted ovarial tissue and eggs). Shell vials containing 2 to 3 ml of media were used for investigating ovarial explants and eggs in liquid. One half of all the media (liquid and agar) had antibiotics incorporated in the following amounts: 6.6 mg/100 ml aretan, 500 μg/ml penicillin, 125 μg/ml streptomycin. Of a number of fungicides used for axenization of tissue, preliminary tests indicated that aretan (Ethoxyethyl mercury chloride) was least harmful to the animals.

The nematodes used in the study were immersed for 16 hr in seawater containing 13.3 mg/100 ml aretan, 1000μg/ml penicillin, 125 μg/ml streptomycin. Dissections of the ovarial explants were also conducted in this solution. Each substrate containing eggs, ovarial explants or whole animals was replicated three times.

For a more detailed assessment of the effect of the sterilizing mixture on the culturing of whole animals and eggs the antibiotic solution was separated into its component parts and tested. The experimental design to test any reaction was as follows: (i) seawater, (ii) penicillin 1000 μg/ml, (iii) streptomycin 125 μg/ml, (iv) aretan 13.3 mg/100 ml, and (v) the combination of all. Each treatment was replicated four times. All material was stored at 15 C and examined upon completion of experimental set-up and thereafter at weekly intervals.

Results

In evaluation of tissue state, cellularity refers to a condition indistinguishable from the *in vivo* state, i.e. preservation of cell outlines, discrete nucleus and nucleolus.

TABLE 2. Suitable media as indicated by favorable egg, nematode and explant reaction of *D. californicum*.

	Eggs				Whole animals		Gonads			
Media	Liq.	Liq. + A	Agar	Agar + A	Agar	Agar + A	Liq.	Liq. + A	Agar	Agar + A
Seawater	+		+		+	+				
Med. B (SW)			+			+				
C-G (SW)	+		+		+	+				
A-1 (SW)	+		+			+				
199 (SW)					+	+				
26c (SW)					+	+				
Mosquito (SW)					+	+				
K-6								+		+
K-6 (SW)							+		+	+
Bombyx							+	+		
Bombyx (SW)										
Samia (SW)							+	+	+	+
Grace (SW)							+	+	+	+

Response of a magnitude expressable in mathematical terms for growth of the ovarial explants was not obtained; therefore, interpretation was dependent upon observable changes in condition.

The criteria for eggs and whole animals was much more distinct. Egg response was measured by the extent of cell division and development. Whole animals were judged by activity and condition of internal organs and contents.

Table 1 summarizes ovarial explant reaction. Arbitrarily media can be divided into those which supported the entire gonad and those which supported either the ovary or oviduct with included ova. Of those media which supported the entire explant, only two [Samia (SW) and Grace (SW)] showed consistent maintenance under all conditions. In two replicates of Grace (SW) agar and in one replicate of Grace (SW) liquid plus antibiotics, cellularity was maintained for 8 months. None of the other media approached this degree of maintenance. The remaining satisfactory media, Bombyx, K-6

TABLE 3. Egg development and cleavage of *D. californicum* recorded in days.

Media	Liquid		Liquid + A		Agar		Agar + A	
	Balled	Cell division	Balled	Cell division	Balled	Cell division	Balled	Cell division
Control (SW)	+	larva	+	0	+	larva	+	0
Bombyx (SW)	0	0	0	0	0	0	0	0
Bombyx	+	0	+	2	+	0	0	0
Samia (SW)	0	0	0	0	0	0	0	0
Samia	+	0	0	0	0	0	0	0
A-1 (SW)	+	32	+	0	+	8	+	0
A-1	0	0	+	0	0	0	0	0
K-6 (SW)	+	0	+	0	+	0	+	0
K-6	+	0	+	0	+	0	+	0
C-G (SW)	+	64	+	0	+	8	+	0
C-G	+	0	+	0	+	2	+	0
Mosquito (SW)	+	0	+	0	+	0	+	0
Mosquito	+	0	+	0	+	0	+	0
26c (SW)	+	0	+	0	+	2	+	0
26c	+	0	0	0	+	0	+	0
Media B (SW)	+	2	0	0	+	16	+	0
Media B	+	0	0	0	0	0	0	0
199 (SW)	+	0	+	0	+	2	+	0
199	0	0	0	0	0	0	0	0
Grace (SW)	+	0	0	0	+	0	+	0
Grace	0	0	+	0	0	0	0	0

(SW), and K-6 did maintain to a lesser degree the entire explant (Table 2).

Additional promising media, K-6, C-G (SW), 26c (SW), media B, A-1, and Bombyx (SW) maintained cellularity in only a portion of the explant (Table 1). K-6 agar and C-G (SW) agar maintained the ovary, whereas the other "promising media" maintained cellularity of the ova within the oviduct.

Grace (SW), Samia (SW), K-6 (SW) and K-6, in addition to preserving cellularity for 14 days, also preserved cell outlines for an additional 7-day period of observation. The other media considered as "successful" or "promising" did not consistently preserve the ovarial explant cellular structure at the end of 21 days. The final area for loss of cellularity, after deterioration began, was in those cells at the junction of the ovary and oviduct.

Once deterioration of cellularity had begun, a dark unidentified exudate emanated from the oviduct and eggs in Bombyx (SW), Bombyx, Samia, K-6 (SW), K-6, Mosquito, Media B and 199 (SW). In Grace liquid plus antibiotics and in Grace agar plus antibiotics the tissues and eggs were colored red. Crystallization of the gonads occurred only in Media 26c and 26c (SW).

Eggs in the seawater media minus antibiotics showed the greatest development. In the case of seawater agar, cleavage and development were followed from the single "ball" cell stage to the hatched larva. No development was noted in any media containing antibiotics with the single exception of one egg in Bombyx that attained the two-cell stage (Table 3). Of the defined media only A-1, C-G and Media B, all with seawater replacements, showed any significant egg development. The maximum cleavage divisions recorded (64 cells) were in C-G seawater liquid. Table 3 indicates that in several media the balled single cell stage was initially maintained. This balling of egg

TABLE 4. Survival in days of whole animals of *Deontostoma californicum* in culture.

	No antibiotics				Antibiotics			
	Days active	Days quiescent	Total activity	Moulting larvae	Days active	Days quiescent	Total activity	Moulting larvae
Seawater (SW)	24	–	24		24	–	24	
Bombyx (SW)	3	0[a]	3		3	0	3	
Bombyx	3	0	3		3	0	3	
Samia (SW)	3	0	3		3	0	3	
Samia	3	0	3		3	0	3	
A-1 (SW)	3	0	3		17	7	24	1
A-1	3	0	3		3	0	3	
K-6 (SW)	3	0	3		3	0	3	
K-6	3	0	3		3	0	3	
C-G (SW)	24	–	24		24	–	24	1
C-G	3	0	3		3	0	3	
Mosquito (SW)	10	14	24	1	10	14	24	
Mosquito	3	0	3		3	0	3	
26c (SW)	10	14	24		10	7	17	
26c	3	0	3		3	0	3	
Media B (SW)	3	4	7		24	–	24	2
Media B	3	0	3		3	0	3	
199 (SW)	17	7	24		17	7	24	
199	3	4	7		3	4	7	
Grace (SW)	3	0	3		3	0	3	
Grace	3	0	3		3	0	3	

[a] (0) = Dead.

content is an essential preliminary step to cleavage of the egg of *Deontostoma*. Media K-6 and K-6 (SW) caused an unusual swelling of the egg shell.

Whole animals were observed for 24 days. At the end of this period the adult nematodes in seawater had cleared and resorbed the gonadal contents. The same condition prevailed in A-1 (SW), C-G (SW), Mosquito (SW), 26c (SW), Media B (SW) and 199 (SW). In all cases prolonged survival took place only in those media in which the inorganic salts had been replaced by seawater. Of the four media in which larvae moulted, only Mosquito (SW) did not contain antibiotics (Table 4).

The reaction of whole animals and eggs to antibiotic treatment, as individual constituents and in combination, was compared with nematode activity in seawater. Adult nematodes in penicillin and streptomycin reacted comparably to those maintained in seawater throughout the days of observation. At the termination of the observation period all nematodes were active. The nematodes survived 14 days in the combination of penicillin, streptomycin and aretan. Exposure to aretan alone resulted in death within 7 days.

In seawater without antibiotics and in seawater containing penicillin, eggs did not continue cleavage past 14 days. The addition of streptomycin to seawater maintained eggs for 21 days. Egg cleavage was inhibited within 7 days when in aretan and combined antibiotics.

DISCUSSION

For purposes of comparison and to note trends in the physical and chemical effects of classes of compounds on nematodes, it was necessary to standardize media constituent concentrations. Media composition was calculated in terms of solute moieties (mM). Salts were converted to ionic expressions and along with other constituents ex-

pressed in mM. It was not possible, however, to obtain a complete analysis of yeast extract (Yeastolate) and lactalbumin hydrolysate. A partial knowledge of constituents allowed a tentative judgment regarding the relative efficacy of media to support growth. Any attempt to abstract from the recorded data has the inherent property of compromise. This became a necessity in order to evaluate salts, sugars, amino acids, organic acids, and vitamins.

In attempting to understand the responses of *D. californicum* in the artificial environment of culture media, data could not be directly compared with that of soil or freshwater nematodes. In addition to media response differences which could perhaps be anticipated between explant tissues and life stages, differential responses also existed between the life stages. The results indicated that certain stages could have characteristic demands. If the life cycles of animal parasitic and obligate plant parasitic nematodes are considered, one could have expected the diverse reaction exhibited by *D. californicum.* For example, animal parasites and the obligate plant parasites are known to require absolute environmental changes in some or all of their life stages. Soil-dwelling nematodes by the nature of their environment indicate a wide tolerance for change because of the unpredictable fluctuations that occur in their normal environment. The apparent anomaly comes with the marine nematode which is for the most part subject to a constant environment at all stages. Animal parasitologists have recognized that the culture medium is of necessity an artificial environment. It is then apparent that the axenic culture of nematodes can be influenced by the nature of the life cycle. Nematodes tolerating a wide range of environmental fluctuation are less demanding than obligate parasitic forms. *D. californicum* is intermediate; though adapted to its natural environment, when placed on artificial media this adaptation is not suitable and as such the animal has more rigid demands.

It would seem that marine nematodes adapted to a constant environment would be suited for axenic culture. However, the deposited egg is shielded from the intrusion of particulate matter by a selectively permeable membrane. In culture, the nutrients are being supplied in solute form. On the other hand, soil nematodes apparently are adapted to a naturally fluctuating solute environment.

The data in Table 2 summarizes the categories representing the most promising of the media tested. It was evident with eggs that addenda to media are not required, indeed they may be harmful. The levels of glutamic acid and glycine present in media C-G have only a slight effect. It would also appear that amino acids at concentrations present in media A-1 play a detrimental role. Media with low levels of addenda and seawater substitution (C-G and A-1) most closely approximated the eggs natural environment and did allow some developmental response. Therefore, it is concluded that any attempt to culture marine nematodes must take into consideration that the egg stage should be maintained in an environment of seawater. It may be that any external supplemental nutrition is detrimental to development. Addenda may have a harmful effect from the diffusion of toxic levels of chemicals into the egg.

Deontostoma californicum, adults and larvae, will tolerate a wider range of addenda to media than the eggs. Media B (SW), and media C-G (SW) are, in their support of the whole animal, comparable to seawater. The addition of maltose, glucose, glutamic acid or glycine did not appear to be detrimental to survival; neither did they give evidence of any nutrient support.

The activity (days active) of adults and larvae in media A-1 (SW) plus antibiotics and 199 (SW) with and without antibiotics were comparable but somewhat less than in seawater (SW) without addenda (17 days: 24 days). This manifested negative effect on activity of media 199 with antibiotic and A-1 with antibiotic may have been due to the increased levels of amino acid addenda. However, the difference between the media could be due to low levels of vitamins in medium 199 which may aid survival, while in A-1 the unidentified constituents in lactalbumin hydrolysate may hinder it. Because of heavy contamination, A-1 (SW) without antibiotics could not be used to assess constituent effects.

The observed longevity with media 26c (SW) and Mosquito (SW) was 10 days. The amino acid concentrations of media 26c are of the same order as media 199, but vitamin concentrations are approximately one-hundred times greater. Ascorbic acid, a strong reducing agent, is very high in media 26c (5.68 millimoles/liter); this constituent was not present in other media tested. The levels of amino acid in Mosquito media are higher than in media 26c or 199 but lower than media Samia, Bombyx, Grace or K-6. Therefore, it appears that the threshold for amino acid concentrations might be between the levels represented by media Mosquito and 26c. Of those media which proved useless Samia is low in amino acids but high in organic acids; Bombyx and Grace are high in amino acids and in organic acids; K-6 contains no organic acids and is low in amino acids but still higher than the suspected threshold.

Those media which showed the greatest promise for the support of the explanted tissues illustrate that gonads have a need for a wide range of nutritive components. Scattered instances of short term maintenance (Table 1) of the oviduct contents may be explained in part by the nature of the organ rather than the nutrient value of the media. In media B and A-1 where addenda are low the apparent maintenance may be due to age of ova. The older ova being similar to fertilized eggs do not require and, in fact, are hindered by high substituent levels. The ova in these instances would have different characters than the explanted ovary itself.

Other than these scattered apparent inconsistencies the explanted tissues demonstrated clearly that a more complete complement of media constituents at moderate levels is required. Extrapolating from those promising media of Table 2 it can be deduced that vitamins (K-6 and K-6 SW) or organic acids (Samia SW, Bombyx) are beneficial; however, either is less satisfactory than a combination (Grace SW). Balanced high salt content appears necessary, seawater-like solutions being the most satisfactory. However, high salinity only is of no value. The successful media would indicate the necessity of organic substituents with moderate concentrations of amino acids on the order of those incorporated in Grace and salts of the order of seawater.

Culture procedures with free-living nematodes, because of the nature of their food habits and stomodeal structures, necessitate not only surface sterilization but also sterilization of the alimentary canal. A not uncommon fungicide recommended for this purpose is aretan. The possible influence of any antibiotic treatment as a factor detrimental to survival cannot be ignored. The influence of the continuous exposure of nematodes, eggs or explanted tissues on media in which aretan was incorporated is documented in Tables 1, 2, 3, and 4. The degree of influence where antibiotics were not incorporated in the media is not clear because all nematodes had been exposed to antibiotics for surface and internal steriliza-

tion prior to placement on media. It was evident that antibiotics permeated the body by the red staining of the intestinal cells by aretan. On Grace's media, with incorporated antibiotics, the explanted tissues and eggs absorbed and concentrated aretan to such an extent that they were visibly red.

The effect of antibiotics must be considered and the aretan employed for its fungicidal action was clearly injurious to the whole animal as well as explants. The observations in these experiments on maintenance and survival of debilitated nematodes and tissues provide guidelines for eventual satisfaction of the nutritional requirements, though ultimate success in nematode culture lies in the definitive evaluation of media constituents and concentrations as well as the employment of a less injurious fungicidal agent.

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