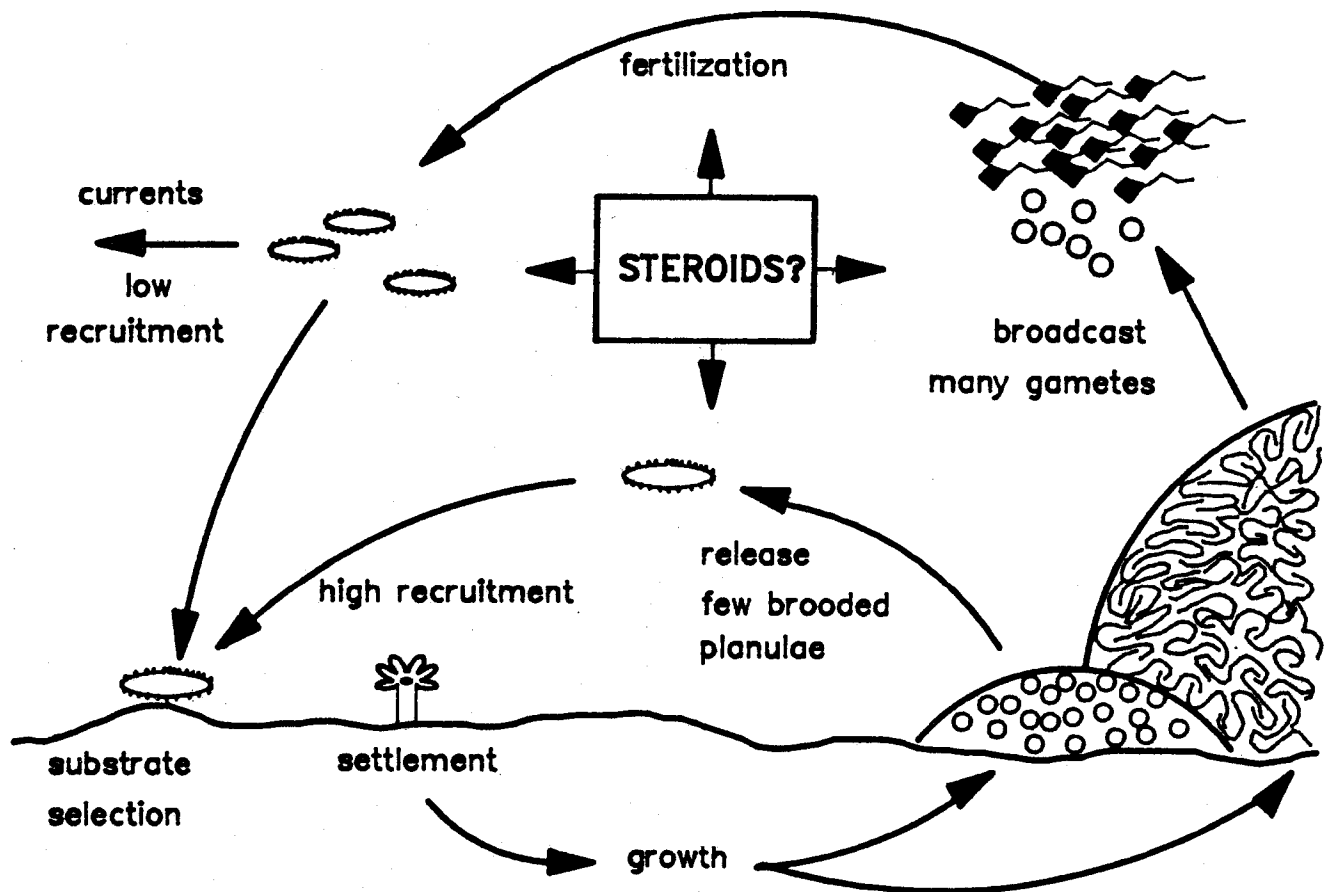


Aspects of the Steroid Biochemistry of the Scleractinian Coral, *Favia fragum*

A Dissertation by

Nancy J. Gassman



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UNIVERSITY OF MIAMI

ASPECTS OF THE STEROID BIOCHEMISTRY OF THE
SCLERACTINIAN CORAL, *FAVIA FRAGUM*

By

Nancy J. Gassman

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida

May, 1992

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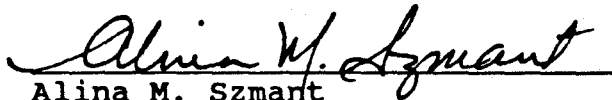
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Aspects of the Steroid Biochemistry
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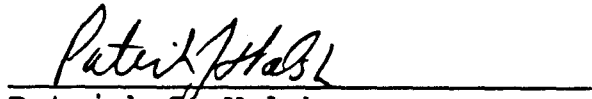
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Coral	163	164	165	166	168	pellet 127	planula
Day ANM	8	8	8	11	11		
Retent (min)							
55.09							
56.87	7491	5818			25254	9774	9530
60.09	65430		20518	50213	17259		29753
61.09					30565		
62.41	61301	99577	247030		19034	125670	526310
65.31							26450
66.19						24197	29971
68.9							13541
69.51			28424				43651
69.87	10108	17404		7436	22783	14267	
72.48							
77.99			53805		27447		85558

GASSMAN, NANCY J. (Ph.D., Marine Biology and Fisheries)

Aspects of steroid biochemistry in the scleractinian coral,
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No. of pages in text 158

The steroid biochemistry of the scleractinian coral, *Favia fragum* (Esper), was studied in an attempt to elucidate the role of these compounds in the regulation of reproduction of corals. *F. fragum* exhibits a lunar periodicity in all phases of gametogenesis, embryogenesis and planulation. The timing of its reproductive events is similar among many Caribbean sites. However, in the south Florida population of *Favia fragum*, the peak in planulation is delayed by several days and fewer planulae are produced in comparison to the Puerto Rican population, possibly due to latitudinal differences in temperature or photoperiod.

Since cytochrome P-450 and associated mixed function oxidase (MFO) enzymes are important in the metabolism of steroids as well as xenobiotics, coral tissue was assayed for the activity of these enzymes over the lunar cycle. The cytochrome P-450 content and the activities of one cytosolic enzyme and three microsomal enzymes are reported for the first time in cnidarians. In general, the MFO activity is low when compared to other invertebrates and vary greatly over the

Coral	163	164	165	166	168	pellet 127	planula
Day ANM	8	8	8	11	11		
Retent (min)							
35.22	6761	8449		2127	6302	9216	25561
35.71							
36.51						17415	3338
36.88	13077	13157	5226	3734	16493	24303	6939
37.24	10406	9970	6548	2819	12669	14661	4825
37.4	18694	21887	17188	13640	20602	25233	47931
37.87					41452	15682	11209
38.06						16219	
38.41	17769		28632	31505		28272	12476
38.66						19663	8640
38.94	7961	6958			12517	13968	5616
39.08				15801			8939
39.26						12979	
39.65	17175	20764	15131	11874	12517	28211	53220
39.98	14844	12669	25733	36963	17283	33774	44095
41.21							
42.56	3056						
42.81							
43.38						7289	
43.76							
43.88							
44						11607	
44.36						11770	
44.64							8780
44.91	19684	21549	7923	6513	16304	21553	49847
46.06						4169	
46.83							
46.96	6652					10457	4234
47.34							
48.19	13170	15138	8431	7036	11588	8759	38170
48.79	9597	15180	8735	12092	18008	4622	14291
50.48							
50.66						2465	
51.19							13270
52.12	7745	10556	8128		6640	6034	30911
52.6	5437				5366	9770	
53.38							

lunar period and among colonies with no apparent pattern. Coral animal tissue homogenates as well as whole colonies incubated with radiolabelled progesterone produced many metabolites including testosterone, presumably through the MFO enzyme system. This demonstrates the ability of corals to take up and metabolize steroids.

Sterol profiles of coral tissues were examined over the lunar cycle to determine whether there were cyclic patterns in their presence that might be correlated with reproductive events. Many compounds were present only at specific times over the lunar period, but the cyclical nature of their presence could not be confirmed.

Although this dissertation has demonstrated the ability of the coral, *Favia fragum*, to take up and process steroids possibly through cytochrome P-450 mixed function oxidases, the role of steroids in the regulation of reproduction in corals remains an open question and fertile territory for future research.

Coral Id	153	154	155	156	157	158	159	160	161
Day ANM	20	23	23	23	0	0	0	4	4
Retent (min)									
55.09									
56.87	42235	11263		29229	17152		3592	49617	
60.09	28188		13746	62224	34000			9709	67539
61.09	24889			23396				55815	
62.41	271760	90224	42421	601640	465960	24982	41497	106730	161330
65.31	17151			36559	24705				
66.19		7900							
68.9				14488	16029				
69.51	14055			42129	84699				
69.87	27805	28249				7644	10834	26241	24611
72.48				9683					
77.99	73504			132010	97988		3440	35470	48199

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Only one name is printed on the binder of this document, but countless people contributed to its contents. I wish to thank my advisor, Dr. Alina Szmant, for providing the opportunity to do research around the world, for giving me an office with a great view and better microscope, and for being supportive for the duration of this dissertation. In addition to my committee members (see title page), I thank Drs. D. Baden, J. Fell, E. Saltzman and J. Brown for their advice and access to their equipment and staff as well as Dr. L. Bunkley-Williams for sending surf temperature data for Puerto Rico.

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Coral	136	137	138	141	143	144	145	146	152
Day ANM	10	10	10	15	15	17	17	17	20

Retent (min)

55.09				37277					
56.87	3716	39001	28461		72483	10723	7140	4311	9429
60.09			14134	34677	22636	14172		9721	26465
61.09		21856			56834				
62.41	28171	91670	126760	55859	171010	198190	34242	38511	168750
65.31						15583			
66.19	10280		23124			16946			16677
68.9									
69.51									
69.87	8687	14864	30176	15455	27146	23758	7694	10170	17223
72.48									
77.99			55975		36604	21374			34591

DEDICATION

To my parents:

Prof. Paul Gassman who is a swell guy
and Gerda Gassman who keeps him from swelling too much

Coral	136	137	138	141	143	144	145	146	152
Day ANM	10	10	10	15	15	17	17	17	20
Retent (min)									
35.22	15070	14142	37084	13360	32846	17223	23323	25372	29340
35.71								2372	
36.51	2579		10346			9836			10423
36.88	9776	16770	24957	22687	24733	4177	10468	7270	15169
37.24	5351	11403	14975		11793		7349	4761	8101
37.4	31133	31589	63160		65216	31422	43034	45276	54256
37.87			10885	30758					
38.06			8524			11612	9353		
38.41		19711	15398		37233				
38.66			16242		11291				10673
38.94	6797	7441	9309		6741	12034	4411	6503	5561
39.08		6594	17551	14653	7246	11411			46292
39.26									
39.65	33794	31394	65708	42666	80662	41424	46160	51790	64020
39.98	222950	159800	133930	73514	50781	34404	24927	21920	49605
41.21			2357	139240		445475			
42.56			7295						
42.81									
43.38			9672						
43.76									
43.88									
44.00			7919						
44.36									
44.64			8126		5442			7229	6143
44.91	33876	36100	63009	29618	83212	35727	37499	45758	50405
46.06									
46.83									
46.96			7404		4936			5432	6048
47.34									
48.19	23209	20880	45600	19474	58237	26621	23280	23436	35934
48.79				10609		4374			9320
50.48									
50.66									
51.19		16222	19065		8559				
52.12	16176	17807	32692	10363	43380	19213	15096	23436	25752
52.6								5130	
53.38				13589					

TABLE OF CONTENTS

PAGE

Acknowledgements.....	iii
List of Figures.....	viii
List of Tables.....	x
CHAPTER 1 - Introduction.....	1
CHAPTER 2 - The timing of gametogenesis and planulation in the scleractinian coral, <i>Favia fragum</i> , from south Florida.....	12
Material and Methods.....	15
Results.....	17
Discussion.....	23
CHAPTER 3 - Cytochrome P-450 content and xenobiotic metabolizing enzyme activities in the scleractinian coral, <i>Favia fragum</i> (Esper).....	28
Material and Methods.....	31
Results.....	37
Discussion.....	42
CHAPTER 4 - Metabolism of progesterone by scleractinian corals.....	48
Material and Methods.....	50
Results.....	57
Discussion.....	65
CHAPTER 5 - Monthly variation in the sterol content of the scleractinian coral, <i>Favia fragum</i>	71
Material and Methods.....	72

Coral	117	118	119	120	121	126	127	128	129
Day ANM	24	24	27	27	27	3	3	3	6
Retent (min)									
53.38	80126								
55.09						5514			
56.87	19381	38839	45968	7528	7718	31366	40949	5038	38526
60.09	4467	6824	10559	19216	21651				
61.09					20920				27149
62.41	30225	44568	51045	96507	131550	14098	68816	27708	32125
65.31									
66.19	16946		7356	5411		10075		25273	
68.90									
69.51			22483						
69.87	45860	28199	15604	32812	38111	12751		8579	8572
72.48					25988				
77.99	17698	9778		13365	27064				

PAGE

Results.....77

Discussion.....107

CHAPTER 6 - Future directions.....116

Literature Cited.....121

Appendix.....135

Table A.2 Chromatographic data from 1989 lunar sampling. Integrated peak areas of 1-2 ul injections from 200 - 500 ul dilution of total sterols are shown.

Coral	117	118	119	120	121	126	127	128	129
Day ANM	24	24	27	27	27	3	3	3	6
Retent (min)									
35.22	8566	44322	8579	10490	9755	131380		13571	14253
35.71			4496	4404	5501	2109			
36.51	17507		11136			3473			
36.88	25989	40803	16781	24397	27549	10384	3135	11182	19591
37.24	27748	4687	10879	10870	17405	6241		2801	14910
37.40	27809	64446	22059	25261	24674	192530	4641	24296	19868
37.87									
38.06									
38.41	78414	6464	16281			11546		5435	
38.66	230890	13616	28210	15468	18436				
38.94		10657	18843	13853	16512	17584		2703	14625
39.08							43895		
39.26									
39.65	65172	96653	33179	32292	34161	206810	9188	26158	36930
39.98	64573	7273	26465	31213	29232	36055	10075	17846	35725
41.21	42382	220630		4101	5792	19747			
42.56		66596	9698	5492	6307				
42.81									
43.38		3568	23921						
43.76						5042			
43.88						15520			
44.00	60365	6174			3870	11020			
44.36									
44.64		34321							
44.91	65827	83491	15569	23525	17379	136870		22142	17260
46.06									
46.83	212980	36678				4048			
46.96	6372	6087	9627	8687	10257	14958			
47.34						12215			
48.19	10768	66323	13096	15756	14938	93107		14429	13495
48.79	12354	4452	6466	17625	25699				
50.48						3660			
50.66						7926			
51.19	72983					7046			
52.12	22683	55320	9354	11117	10776	55389		10240	9202
52.60	7783		13292	8683	10471				

LIST OF FIGURES

FIGURE	PAGE
1.1 Biochemical pathway from lanosterol to the mammalian reproductive steroids.....	7
2.1 Monthly average surf temperature for Miami Beach, Fl. and Mayaguez, Puerto Rico.....	14
2.2 Summary of the reproductive cycle of <i>Favia fragum</i>	19
2.3 Planulae released per colony of <i>Favia fragum</i>	22
3.1 A typical carbon monoxide difference spectrum of dithionite-reduced microsomes from <i>Favia fragum</i>	37
3.2 Lunar time course of cytochrome P-450, microsomal and soluble protein in <i>Favia fragum</i>	40
3.3 Lunar time course of the enzyme activities of <i>Favia fragum</i>	41
4.1 Diagrammatic representation of two-dimensional TLC of sterol standards.....	58
4.2 Diagrammatic representation of two-dimensional TLC of radiolabelled metabolites of ¹⁴ C-progesterone.....	59
4.3 The distribution of total radioactivity in each metabolite produced by coral tissue homogenates incubated <i>in vitro</i> with ¹⁴ C-progesterone.....	60
4.4 Diagrammatic representation of two-dimensional TLC of radiolabelled metabolites of ¹⁴ C-progesterone following <i>in vivo</i> incubation of the scleractinian coral <i>Favia fragum</i>	66
5.1 1988 <i>Favia fragum</i> sampling chromatographic data expressed as cholesterol units per cm ² surface area..	83
5.2 1988 <i>Favia fragum</i> sampling chromatographic data expressed as cholesterol units per mg protein.....	88
5.3 1988 <i>Favia fragum</i> sampling chromatographic data expressed as percent of total peak area.....	93

Retention Time	%total peak area	4/6/88		
	1	3	4	5
33.03	1.118	1.359	2.462	3.24
34.37	0	0	0	0
36.80	0	0.076	0.056	0.3
38.33	0.078	0.1	0	0
38.70	0	0	0	0
39.53	0	0	0	0
39.72	0	0	0	0
41.25	0	0.148	0.155	0
41.90	0.091	0.08	0.092	0
43.51	0	0	0	0
43.70	0	0	0	0
45.50	0.298	0.294	0.286	0.321
46.04	3.389	2.931	3.127	2.708
46.41	0	0	0	0
46.95	0	0.072	0.076	0
47.63	40.693	40.542	38.034	42.558
47.79	0	0	0	0.395
47.88	0.632	0.507	0.586	0
48.39	0.857	0.65	0.659	0.73
48.71	0.332	0.38	0.314	0.388
49.15	4.236	4.58	4.977	0.446
49.43	0	0	0	0
49.57	0	0	0	0
50.08	0	0	0	0
51.31	3.602	3.616	3.912	3.273
51.77	10.183	10.611	10.117	9.707
52.17	0.271	0.274	0.252	0
52.69	0.234	0.273	0.261	0
53.13	0.181	0.194	0.183	0
53.33	0.289	0.123	0	0
53.75	0.296	0.234	0.245	0.49
54.32	0	0	0.832	1.706
55.32	0	0	0.217	0
55.59	0.23	0.329	0.398	0
56.01	1.575	1.312	1.681	1.224
56.72	13.411	11.727	12.517	9.661
56.99	7.074	9.211	9.181	9.392
58.23	0.405	0.318	0.336	0
59.61	0	0	0	0
63.88	10.526	10.058	9.043	13.462

FIGURE**PAGE**

5.4 1989 <i>Favia fragum</i> lunar sampling protein per surface area.....	99
5.5 1989 <i>Favia fragum</i> lunar sampling chromatographic data expressed as percent total peak area.....	100
5.6 1989 <i>Favia fragum</i> lunar sampling comparison of percent total peak area between mean coral values and planulae.....	106
5.7 Compounds proposed for use as analytical standards...	111

Retention Time	Peak area	4/6/88		
	1	3	4	5
33.03	515800	831810	1420000	627980
34.37				
36.80		46674	32409	58141
38.33	35991	61263		
38.70				
39.53				
39.72				
41.25		90344	89407	
41.90	42077	48754	52902	
43.51				
43.70				
45.50	137450	179920	164990	62175
46.04	1562800	1793200	1803300	524840
46.41				
46.95		43844	43935	
47.63	18767000	24807000	21936000	8248000
47.79				76531
47.88	291260	310250	337820	
48.39	395080	397960	380330	141410
48.71	153240	232730	181360	75291
49.15	1953400	2802300	2870700	86520
49.43				
49.57				
50.08				
51.31	1661200	2212600	2256100	634330
51.77	4696400	6492500	5834700	1881200
52.17	124990	167690	145600	
52.69	107840	166900	150770	
53.13	83476	118940	105360	
53.33	133350	74959		
53.75	136640	143210	141220	95048
54.32			479980	330540
55.32			125000	
55.59	105860	201250	229630	
56.01	726170	803010	969490	237140
56.72	6185000	7175700	7219300	1872300
56.99	3262300	5636100	5295300	1820200
58.23	186850	194550	193770	
59.61				
63.88	4854700	6154600	5215400	2609000

LIST OF TABLES

TABLE	PAGE
2.1 Regional comparison of the timing and seasonality of planulation in <i>Favia fragum</i> in the Caribbean.....	15
3.1 Enzyme activities normalized to mg protein and compared by collection site.....	38
4.1 Thin layer chromatography standards.....	56
4.2 Metabolites from <i>in vivo</i> incubation of <i>Favia fragum</i> with ¹⁴ C-progesterone.....	62
5.1 Characteristics of 1988 <i>Favia fragum</i> samples from preliminary sampling.....	80
5.2 Retention time of <i>Favia fragum</i> sterols in the 1988 sampling.....	81
5.3 Characteristics of 1989 lunar sampling of <i>Favia fragum</i>	98
A.1 Gas chromatographic data from 1988 sampling.....	135
A.2 Chromatographic data from 1989 lunar sampling.....	151

Retention Time	cholesterol units/ mg supernatant protein		4/6/88	
	1	3	4	5
33.03	0	0.477	1.034	1.021
34.37	0	0	0	0
36.80	0	0.027	0.023	0.094
38.33	0	0.035	0	0
38.70	0	0	0	0
39.53	0	0	0	0
39.72	0	0	0	0
41.25	0	0.052	0.065	0
41.90	0.037	0.028	0.039	0
43.51	0	0	0	0
43.70	0	0	0	0
45.50	0.119	0.104	0.120	0.101
46.04	1.357	1.029	1.313	0.853
46.41	0	0	0	0
46.95	0	0.025	0.032	0
47.63	16.296	14.229	15.975	13.403
47.79	0	0	0	0.125
47.88	0.253	0.178	0.246	0
48.39	0.343	0.228	0.277	0.230
48.71	0.133	0.134	0.132	0.122
49.15	1.696	1.607	2.091	0.141
49.43	0	0	0	0
49.57	0	0	0	0
50.08	0	0	0	0
51.31	1.443	1.269	1.643	1.031
51.77	4.079	3.724	4.249	3.057
52.17	0.108	0.096	0.106	0
52.69	0.094	0.095	0.110	0
53.13	0.072	0.069	0.077	0
53.33	0.115	0.043	0	0
53.75	0.118	0.082	0.103	0.154
54.32	0	0	0.349	0.537
55.32	0	0	0.091	0
55.59	0.092	0.116	0.167	0
56.01	0.631	0.460	0.706	0.386
56.72	5.370	4.116	5.257	3.043
56.99	2.833	3.233	3.856	2.957
58.23	0.162	0.112	0.141	0
59.61	0	0	0	0
63.88	4.215	3.530	3.798	4.239

CHAPTER 1

INTRODUCTION

Many marine organisms have patterns of reproduction that have the same periodicity as the lunar cycle. Reproductive patterns, which exhibit rhythms correlated to exogenous (or environmental) cues, may have corresponding endogenous factors regulating biochemical events. Steroids and neuropeptides are found throughout the animal kingdom and have been shown to act as regulators of certain biochemical events, particularly those associated with reproduction (Sandor & Mehdi, 1979). Most studies of the reproductive role of these compounds have been conducted in the morphologically, physiologically, and biochemically complex higher vertebrates. Little is known of their role in the regulation of reproduction in phylogenetically more primitive animals, where their regulatory function most likely evolved. The original intent of this research was to investigate the role of steroids as biochemical regulators of key events in gametogenesis in a lower invertebrate, the scleractinian coral, *Favia fragum* (Phylum Cnidaria, Class Anthozoa, Order Scleractinia). However, before this objective could be attained, it was necessary to describe the biochemical processes important for the metabolism of steroids and baseline levels of these

Table A.1d Data for 4/6/88.

Retention Time	cholesterol units/cm ²	4/6/88		
	1	3	4	5
33.03	0	0.396	1.149	0.696
34.37	0	0	0	0
36.80	0	0.022	0.026	0.064
38.33	0	0.029	0	0
38.70	0	0	0	0
39.53	0	0	0	0
39.72	0	0	0	0
41.25	0	0.043	0.072	0
41.90	0.036	0.023	0.043	0
43.51	0	0	0	0
43.70	0	0	0	0
45.50	0.117	0.086	0.133	0.069
46.04	1.33	0.854	1.459	0.582
46.41	0	0	0	0
46.95	0	0.021	0.036	0
47.63	15.97	11.81	17.748	9.141
47.79	0	0	0	0.085
47.88	0.248	0.148	0.273	0
48.39	0.336	0.189	0.308	0.157
48.71	0.13	0.111	0.147	0.083
49.15	1.662	1.334	2.323	0.096
49.43	0	0	0	0
49.57	0	0	0	0
50.08	0	0	0	0
51.31	1.414	1.053	1.825	0.703
51.77	3.997	3.091	4.721	2.085
52.17	0.106	0.08	0.118	0
52.69	0.092	0.079	0.122	0
53.13	0.071	0.057	0.085	0
53.33	0.113	0.036	0	0
53.75	0.116	0.068	0.114	0.105
54.32	0	0	0.388	0.366
55.32	0	0	0.101	0
55.59	0.09	0.096	0.186	0
56.01	0.618	0.382	0.784	0.263
56.72	5.263	3.416	5.841	2.075
56.99	2.776	2.683	4.284	2.017
58.23	0.159	0.093	0.157	0
59.61	0	0	0	0
63.88	4.131	2.93	4.22	2.891

compounds.

Hormones, bioregulators and endogenous factors

The terms biochemical regulator and endogenous signal have been used to describe a chemical which controls reproductive events. A more common term is hormone. As applied to invertebrates, hormones may be defined "as substances secreted in one part of the body and transported to others parts where they evoke, in minute quantities, physiological responses" (Barrington, 1979). However, since cnidarians and other lower animals lack defined circulatory systems and endocrine organs, chemical communication within tissues or cell types may be mediated by substances that do not have a defined origin and that are transported by diffusion. Because the term hormone is usually associated with organs having endocrine function, Sandor and Mehdi (1979) suggested the term bioregulator to indicate a biochemical signal acting at a target site to bring about specific physiological changes.

Bioregulators within the Cnidaria

Several studies document the possible existence of bioregulators within the Cnidaria. In the class Hydrozoa, activity of neurosecretory cells in the growth region of hydra during regeneration provides circumstantial evidence for the presence of a growth regulator (Burnett & Diehl, 1964). Gender in hydra may also be controlled by a "diffusible substance" as

Retention Time	%total peak area	3/28/88			
	1	2	3	4	5
33.03	1.831	2.162	1.326	2.168	1.481
34.37	0	0	0.047	0	0
36.80	0.134	0.208	0.084	0.118	0.131
38.33	0	0	0	0	0
38.70	0	0	0	0	0
39.53	0	0	0	0	0
39.72	0	0	0.154	0	0
41.25	0.182	0.123	0	0	0.128
41.90	0	0.156	0.118	0	0.138
43.51	0	0	0	0	0
43.70	0	0	0	0	0
45.50	0.392	0.471	0.394	0.31	0.459
46.04	2.969	4.12	3.156	2.869	3.917
46.41	0	0	0	0	0
46.95	0	0	0	0	0
47.63	41.148	40.054	43.402	42.35	41.496
47.79	0	0	0	0	0
47.88	0.447	0.83	0.457	0.526	0.628
48.39	0.707	0.706	0.619	0.387	0.702
48.71	0.356	0.532	0.489	0.489	0.559
49.15	2.705	5.469	2.994	3.329	5.608
49.43	0	0	0	0	0
49.57	0	0	0	0	0.22
50.08	0	0	0	0	0
51.31	2.527	4	3.799	3.263	4.421
51.77	7.37	9.464	9.403	12.126	10.858
52.17	0	0.273	0.171	0.234	0.238
52.69	0	0.415	0.139	0	0.342
53.13	0.397	0.471	0.402	0.153	0.323
53.33	0.419	0	0	0	0
53.75	0.361	0.217	0.235	0	0.202
54.32	0	0	0	0	0
55.32	0	0	0	0	0
55.59	0.646	0.136	0.716	0.252	0.178
56.01	2.015	1.394	1.582	1.399	1.258
56.72	7.926	13.16	10.571	14.703	12.351
56.99	13.721	4.579	7.988	8.842	4.564
58.23	0.428	0.349	0.283	0.392	0.202
59.61	0	0.596	0	0	0
63.88	13.319	10.116	11.472	8.375	9.596

shown by the gradual sex change of female grafts to male organisms (Brien, 1965). More recent evidence supports the idea that inhibition of head formation on the body stalk of hydra is due to a diffusing substance originating in the head and broken down in the lower part of the body forming a gradient within the organism (MacWilliams, 1983). Within the class Scyphozoa, strobilation events coincide with increased neurosecretory activity, suggesting biochemical regulation of segmentation (Crawford & Webb, 1972). Oocyte maturation in certain jellyfish is regulated by a spawning-inducing substance whose secretion by the ovary is light-induced (Ikegami et al., 1978) providing evidence for bioregulation of gametogenic events coordinated by an exogenous cue.

Steroids as bioregulators in invertebrates

Neurosecretion of neuropeptides and other such substances has been postulated to be the most significant mechanism by which lower animals regulate development and reproduction (Highnam & Hill, 1977). However, the discovery of ecdysone, the arthropod moulting hormone, provided the impetus for the study of steroids as important molecules in invertebrate comparative endocrinology (Sandor, 1980). It is now known that steroids often act as the mediators of events while neuropeptides serve to stimulate the production of steroids. The basic precursor molecule of all steroids, cyclopentoperhydrophenanthrene, has been postulated to be one of the

Retention Time	peak area	3/28/88			
	1	2	3	4	5
33.03	556700	1058400	471160	653710	795610
34.37			16854		
36.80	40760	101690	29743	35432	70380
38.33					
38.70					
39.53					
39.72			54584		
41.25	55220	60061			68712
41.90		76195	41975		74100
43.51					
43.70					
45.50	119120	230790	139940	93523	246600
46.04	902530	2017300	1121100	864990	2104500
46.41					
46.95					
47.63	12509000	19612000	15419000	12769000	22297000
47.79					
47.88	135870	406230	162510	158740	337570
48.39	214960	345840	219930	116600	377380
48.71	108360	260600	173730	147450	300370
49.15	822320	2677600	1063500	1003800	3013400
49.43					
49.57					118140
50.08					
51.31	768250	1958700	1349700	983890	2375700
51.77	2240600	4633800	3340400	3656200	5834400
52.17		133490	60603	70542	127710
52.69		203340	49420		183770
53.13	120710	230840	142860	45988	173480
53.33	127440				
53.75	109710	106400	83536		108340
54.32					
55.32					
55.59	196380	66512	254370	76062	95748
56.01	612440	682440	562200	421850	676050
56.72	2409400	6443500	3755600	4433200	6636600
56.99	4171000	2242000	2837700	2666100	2452600
58.23	130180	170920	100400	118180	108490
59.61		291890			
63.88	4048800	4953300	4075400	2525100	5156300

primordial molecules from which life was created (Sandor & Mehdi, 1979). Steroid precursors may be ancient molecules, pre-dating the eukaryotes, and may have been synthesized abiotically. This hypothesis has been used to explain the omnipresence of steroids in the biosphere (Sandor & Mehdi, 1979).

Metabolism of steroids

The most common sterol, cholesterol, acts as a universal precursor in the pathway to the C_{18} , C_{19} , and C_{21} androgen and estrogen steroids (Figure 1.1), important in the control of reproduction in higher organisms. "The term sterol [is] used to embrace compounds closely related to cholesterol and the term steroid encompasses the multitude of naturally occurring and synthetic compounds which may be viewed as derived from sterols" (Bergmann, 1962). The committed step in the pathway from cholesterol to these steroids is the cleavage of the alkane chain from the fourth ring (C_{17}) of the cyclopentane backbone. One notable exception in which cholesterol is not used as a precursor is found in the synthesis of the steroids equilin and equilenin, in pregnant mares (Bhavnani et al., 1975). This pathway is derived from earlier precursors originating in isopentyl pyrophosphate. Many of the enzymes in the pathway from cholesterol to the androgens and estrogens are cytochrome P-450 dependent mixed-function oxygenases (Figure 1.1). Not all organisms are able to synthesize

Retention Time	cholesterol units /mg supernatant protein		3/28/88		
	1	2	3	4	5
33.03	0.620	0.977	0.662	0.832	0.612
34.37	0	0	0.024	0	0
36.80	0.045	0.094	0.042	0.044	0.054
38.33	0	0	0	0	0
38.70	0	0	0	0	0
39.53	0	0	0	0	0
39.72	0	0	0.077	0	0
41.25	0.061	0.056	0	0	0.053
41.90	0	0.070	0.060	0	0.058
43.51	0	0	0	0	0
43.70	0	0	0	0	0
45.50	0.133	0.213	0.196	0.118	0.190
46.04	1.006	1.862	1.576	1.102	1.619
46.41	0	0	0	0	0
46.95	0	0	0	0	0
47.63	13.948	18.104	21.667	16.259	17.153
47.79	0	0	0	0	0
47.88	0.152	0.375	0.228	0.201	0.260
48.39	0.240	0.319	0.310	0.148	0.291
48.71	0.120	0.241	0.244	0.189	0.231
49.15	0.917	2.472	1.494	1.279	2.318
49.43	0	0	0	0	0
49.57	0	0	0	0	0.091
50.08	0	0	0	0	0
51.31	0.856	1.809	1.897	1.253	1.827
51.77	2.498	4.278	4.693	4.656	4.489
52.17	0	0.124	0.085	0.091	0.098
52.69	0	0.188	0.070	0	0.142
53.13	0.135	0.213	0.201	0.059	0.133
53.33	0.143	0	0	0	0
53.75	0.122	0.098	0.118	0	0.083
54.32	0	0	0	0	0
55.32	0	0	0	0	0
55.59	0.219	0.061	0.357	0.096	0.073
56.01	0.683	0.630	0.790	0.538	0.520
56.72	2.687	5.947	5.277	5.645	5.105
56.99	4.651	2.070	3.988	3.396	1.887
58.23	0.146	0.158	0.141	0.150	0.083
59.61	0	0.269	0	0	0
63.88	4.514	4.572	5.726	3.216	3.967

cholesterol and those that cannot require it as a dietary supplement (e.g. insects, Nes & McKean, 1977).

steroids in Cnidaria

The field of steroid chemistry of the Cnidaria originated with the investigations of Bergmann and his students (Bergmann et al., 1943) into the sterol content of invertebrates and the subsequent discovery of gorgosterol. Gorgosterol, a C_{30} sterol, has been found in many gorgonians (Class Anthozoa, Order Hexacorallia) and other cnidarians with zooxanthellae (including the hard coral *Manicina areolata*) (Ciereszko et al., 1968). Several studies have attributed occurrence of gorgosterol to the presence of zooxanthellae (Ciereszko et al., 1968; Kokke et al., 1981) and have shown that these dinoflagellates may be responsible for the methylation of precursors of gorgosterol (Ciereszko & Karns, 1973). Gorgonians have the ability to synthesize sterols as shown by Popov et al. (1976) who identified 49 sterol components in the gorgonians, *Pseudoplexaura porosa* and *Plexaura homomalla*. Unusual C_{25} -hydroxylated sterols and polyhydroxylated sterols have been found in alcyonaceans (the soft corals) (Moldowan et al., 1974; Tursch et al., 1976; Higgs & Faulkner, 1977; Sjostrand et al., 1981).

Within the Scleractinia, study of steroid content has been much more limited. Ciereszko et al. (1968) demonstrated the presence of C_{27} , C_{28} , C_{29} sterols, and gorgosterol in

Table A.1c Data for 3/28/88.

Retention Time	cholesterol units/cm ²	3/28/88			
	1	2	3	4	5
33.03	0.618	0.893	0.445	0.45	0.575
34.37	0	0	0.016	0	0
36.80	0.045	0.086	0.028	0.024	0.051
38.33	0	0	0	0	0
38.70	0	0	0	0	0
39.53	0	0	0	0	0
39.72	0	0	0.052	0	0
41.25	0.061	0.051	0	0	0.05
41.90	0	0.064	0.04	0	0.054
43.51	0	0	0	0	0
43.70	0	0	0	0	0
45.50	0.132	0.195	0.132	0.064	0.178
46.04	1.002	1.702	1.059	0.596	1.52
46.41	0	0	0	0	0
46.95	0	0	0	0	0
47.63	13.892	16.547	14.56	8.796	16.107
47.79	0	0	0	0	0
47.88	0.151	0.343	0.153	0.109	0.244
48.39	0.239	0.292	0.208	0.08	0.273
48.71	0.12	0.22	0.164	0.102	0.217
49.15	0.913	2.259	1.004	0.692	2.177
49.43	0	0	0	0	0
49.57	0	0	0	0	0.085
50.08	0	0	0	0	0
51.31	0.853	1.653	1.275	0.678	1.716
51.77	2.488	3.91	3.154	2.519	4.215
52.17	0	0.113	0.057	0.049	0.092
52.69	0	0.172	0.047	0	0.133
53.13	0.134	0.195	0.135	0.032	0.125
53.33	0.142	0	0	0	0
53.75	0.122	0.09	0.079	0	0.078
54.32	0	0	0	0	0
55.32	0	0	0	0	0
55.59	0.218	0.056	0.24	0.052	0.069
56.01	0.68	0.576	0.531	0.291	0.488
56.72	2.676	5.436	3.546	3.054	4.794
56.99	4.632	1.892	2.68	1.837	1.772
58.23	0.145	0.144	0.095	0.081	0.078
59.61	0	0.246	0	0	0
63.88	4.496	4.179	3.848	1.74	3.725

Manicina areolata. Kanazawa et al. (1977) investigated the sterol content of 22 species of cnidarians including two scleractinian corals. These studies document the presence of sterols in cnidarians, but the C₁₈-C₂₁ steroids of cnidarians have not been characterized.

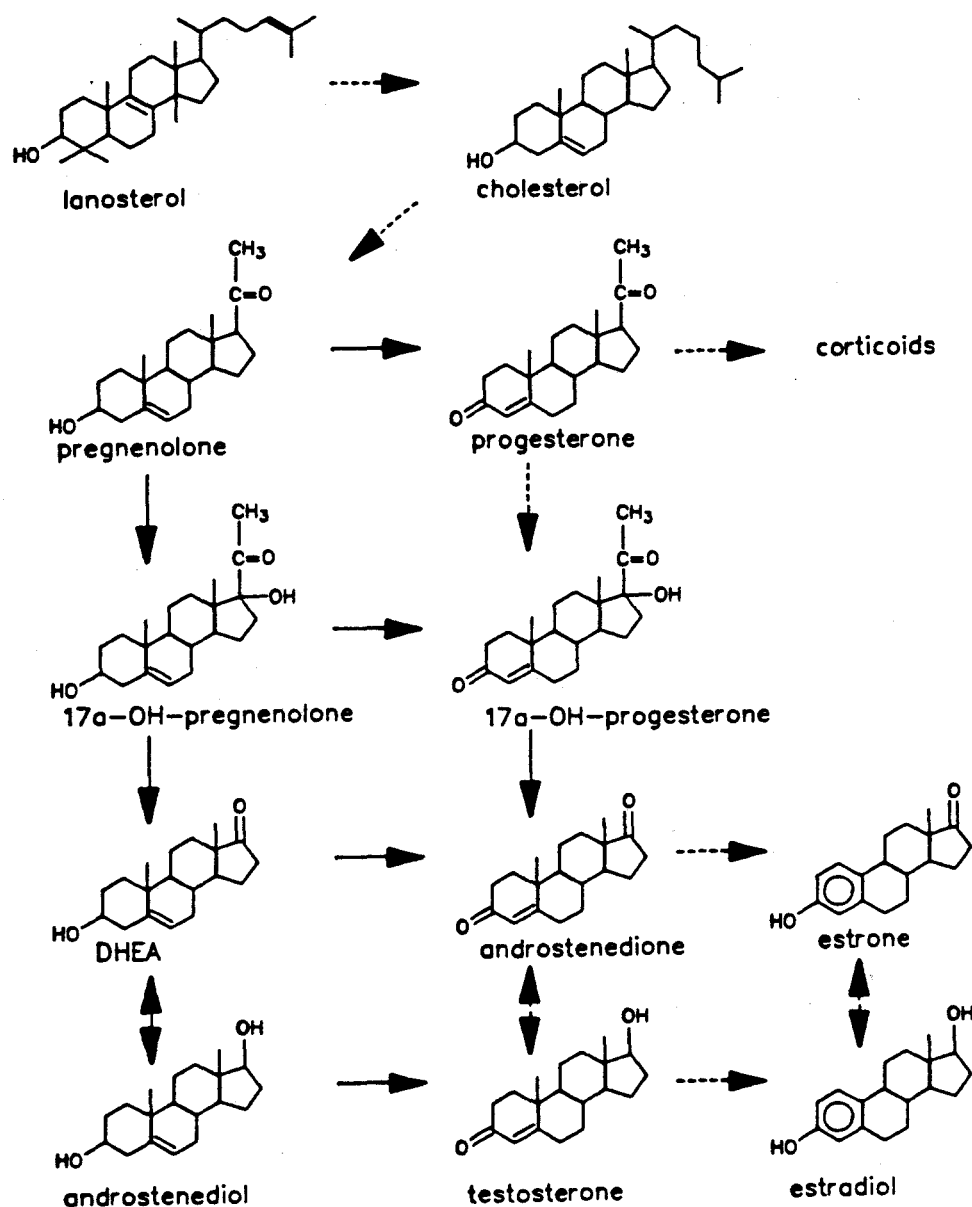
Seasonal variability of compounds associated with reproduction in the Cnidaria has only been documented in a few cases. A single sterol in a californian gorgonian showed greater abundance in late summer, its time of reproduction (Popov et al., 1983). Coll et al. (1989) have shown that eggs from sinularian soft corals contain compounds that are distinct from the parent colony but their function has not been elucidated. Differences between the sterol content of male and female colonies have also been noted in zooanthids (Kelecom & Solé-Cava, 1982).

Reproduction in Scleractinians

Phylogenetically, reproductive structures are first found in the phylum Cnidaria in the form of sex cell aggregates (Hyman, 1940). The Cnidaria are characterized by definite form, radial symmetry, and the development of two distinct tissue layers. Cnidarians are also unique in that they possess elementary neural and sensory systems. These organisms can be used as a model in which to search for the origins of biochemical control of reproduction because they represent an early derivization of the eumetazoan line. The rudimentary

Retention Time	%total peak area	3/22/88	
	1,2,5	3	4
33.03	0	0	0
34.37	0	0.062	0.036
36.80	0.145	0	0
38.33	0	0	0
38.70	0.96	0	0
39.53	0.251	0	0
39.72	0	0	0.042
41.25	0.201	0	0
41.90	0.164	0.126	0.202
43.51	0.225	0	0
43.70	0.21	0	0
45.50	0.51	0.478	0.606
46.04	3.701	3.544	5.277
46.41	0	0	0.128
46.95	0	0	0
47.63	42.569	33.794	34.666
47.79	0	12.298	14.373
47.88	0.584	0.564	0.766
48.39	0.725	0.954	0.759
48.71	0	0.305	0.342
49.15	4.414	3.27	5.484
49.43	0	0.243	0.176
49.57	0	0	0
50.08	0	0.111	0.113
51.31	3.263	3.251	3.277
51.77	8.295	9.301	7.842
52.17	0.223	0.18	0.197
52.69	0	0.287	0.281
53.13	0.332	0.417	0.632
53.33	0	0.381	0.231
53.75	0.259	0.454	0.385
54.32	0	0	0
55.32	0	0	0
55.59	0.377	0.202	0
56.01	1.721	1.115	1.434
56.72	10.665	8.675	13.501
56.99	10.119	4.22	0
58.23	0.346	0.382	0.497
59.61	0	0	0
63.88	9.743	15.384	8.755

Figure 1.1 Biochemical pathway from lanosterol to the mammalian reproductive steroids. Dashed arrows represent cytochrome P-450 dependant mixed function oxygenase enzymes (Norman & Litwack, 1987).



Retention Time	peak area	3/22/88	
	1,2,5	3	4
33.03			
34.37		38438	36817
36.80	43351		
38.33			
38.70	287550		
39.53	75060		
39.72			42943
41.25	60086		
41.90	49151	78370	205670
43.51	67449		
43.70	62772		
45.50	152720	296540	617840
46.04	1108100	2197700	5383000
46.41			130630
46.95			
47.63	12747000	20959000	35362000
47.79		7627300	14662000
47.88	174800	350030	781040
48.39	216960	591850	774260
48.71		189350	348770
49.15	1321700	2028200	5593800
49.43		150710	179380
49.57			
50.08		68677	114950
51.31	977150	2016500	3342900
51.77	2483800	5768500	7999200
52.17	66773	111450	201230
52.69		177950	286610
53.13	99294	258880	645080
53.33		236360	235200
53.75	77447	281640	393000
54.32			
55.32			
55.59	112940	125440	
56.01	515270	691450	1463000
56.72	3193600	5380000	13772000
56.99	3029900	2617400	
58.23	103620	236940	506560
59.61			
63.88	2917500	9541200	8931200

neural and sensory systems of the Cnidaria may provide a means by which biochemical signals can be coordinated and released in conjunction with environmental cues such as the phase of the moon.

Many stony corals (Phylum Cnidaria, Class Anthozoa, Order Scleractinia) exhibit mass synchronous spawning correlated to specific moon phases. Harrison et al. (1984) were the first to document the spawning of 32 species of coral on several nights close to 5 days after the full moon in November, 1982. Subsequent yearly observations have revealed that at least 109 species in the Great Barrier Reef (GBR) participate in epidemic spawning events (Babcock et al., 1986). The biochemical origin of the timing mechanism is not understood. In the Caribbean, while reproduction is usually seasonal, strong ties to a single lunar date among several genera have not been demonstrated (reviewed by Fadlallah, 1983; Szmant, 1986).

While several studies have investigated environmental factors affecting planulation and spawning, few have concentrated on understanding the biochemical regulation of gametogenesis. Jokiel et al. (1985) have shown that night irradiance acts as the synchronizing cue for the lunar cycle of planulation in the Pacific coral, *Pocillopora damicornis*. Babcock (1984) demonstrated the ability to alter spawning time by using varying diel light regimes in *Goniastrea* sp., another Pacific species. Spawning time in *Montipora digitata* can be

Retention Time	cholesterol units /mg supernatant protein		3/22/88
	1,2,5	3	4
33.03	0	0	0
34.37	0	0.025	0.056
36.80	0.017	0	0
38.33	0	0	0
38.70	0.114	0	0
39.53	0.030	0	0
39.72	0	0	0.065
41.25	0.024	0	0
41.90	0.019	0.052	0.313
43.51	0.027	0	0
43.70	0.025	0	0
45.50	0.060	0.199	0.942
46.04	0.439	1.478	8.200
46.41	0	0	0.199
46.95	0	0	0
47.63	5.052	14.102	53.869
47.79	0	5.132	22.335
47.88	0.069	0.235	1.190
48.39	0.086	0.398	1.179
48.71	0	0.127	0.531
49.15	0.524	1.364	8.521
49.43	0	0.102	0.273
49.57	0	0	0
50.08	0	0.046	0.175
51.31	0.387	1.357	5.093
51.77	0.984	3.881	12.185
52.17	0.026	0.075	0.306
52.69	0	0.120	0.437
53.13	0.040	0.175	0.983
53.33	0	0.159	0.359
53.75	0.031	0.189	0.599
54.32	0	0	0
55.32	0	0	0
55.59	0.044	0.085	0
56.01	0.204	0.465	2.229
56.72	1.266	3.620	20.979
56.99	1.201	1.761	0
58.23	0.041	0.159	0.771
59.61	0	0	0
63.88	1.157	6.420	13.606

delayed up to one week by keeping the colonies in constant light and transferring them into a natural L-D light regime (Miller, 1986). These three examples suggest that exogenous light cues somehow regulate the physical events of planulation and spawning. None of these researchers attempted to study the effects of these cues on the biochemistry which regulates maturation events of gametogenesis.

Favia fragum

Favia fragum is a Caribbean species found off the coast of south Florida, the Bahamas, Bermuda and the West Indies (Walton-Smith, 1971), where it is abundant in most shallow reef environments. This species is relatively resistant to the deleterious effects of sediment, fluctuating temperatures, and exposure to low tides (Walton-Smith, 1971). *F. fragum* has a different reproductive pattern than the GBR corals which broadcast all gametes in a single reproductive effort. In this hermaphroditic coral, the eggs are believed to be internally fertilized rather than being shed into the water column where external fertilization takes place. *F. fragum* exhibits lunar periodicity in all phases of gametogenesis, in embryogenesis, and in planulation in Puerto Rico (Szmant-Froelich et al., 1985).

Outline of the dissertation

Chapter 2 compares the timing of gametogenenic events and

Table A.1c Data for 3/28/88.

Retention Time	cholesterol units/ cm ²	3/22/88	
	1,2,5	3	4
33.03	0	0	0
34.37	0	0.021	0.049
36.80	0.015	0	
38.33	0	0	
38.70	0.1	0	
39.53	0.026	0	
39.72	0	0	0.057
41.25	0.021	0	0
41.90	0.017	0.043	0.274
43.51	0.024	0	0
43.70	0.022	0	0
45.50	0.053	0.164	0.824
46.04	0.387	1.218	7.175
46.41	0	0	0.174
46.95	0	0	0
47.63	4.451	11.62	47.135
47.79	0	4.229	19.543
47.88	0.061	0.194	1.041
48.39	0.076	0.328	1.032
48.71	0	0.105	0.465
49.15	0.462	1.124	7.456
49.43	0	0.084	0.239
49.57	0	0	0
50.08	0	0.038	0.153
51.31	0.341	1.118	4.456
51.77	0.867	3.198	10.662
52.17	0.023	0.062	0.268
52.69	0	0.099	0.382
53.13	0.035	0.144	0.86
53.33	0	0.131	0.314
53.75	0.027	0.156	0.524
54.32	0	0	0
55.32	0	0	0
55.59	0.039	0.07	0
56.01	0.18	0.383	1.95
56.72	1.115	2.983	18.357
56.99	1.058	1.451	0
58.23	0.036	0.131	0.675
59.61	0	0	0
63.88	1.019	5.29	11.905

seasonality of planulation of *Favia fragum* in South Florida to the reproductive cycle originally documented in Puerto Rico (Szmant-Froelich et al., 1985). While many spawning events are correlated with a particular moon phase, the reproduction of many marine organisms is often seasonal, following cues such as photoperiod or temperature. Variation in these parameters between sites at higher and lower latitudes may be reflected in the total reproductive effort of *F. fragum*, or it may be surmised that environmental cues which fluctuate over long time periods have less of a role in the regulation of its gametogenic events.

The third chapter investigates the presence of cytochrome P-450 and cycles of enzymatic activity important in steroid as well as xenobiotic compound metabolism. Little is known of the enzymatic capability of these organisms beyond enzymes important in calcification (Streamer, 1980), oxygen detoxification (Dyken & Shick, 1982; Dyken, 1984; Lesser et al., 1990), and those used in electrophoretic studies investigating population genetics (Knowlton et al., 1992).

Chapter 4 summarizes data which illustrate the ability of corals to take up steroids from sea water and metabolize them in *in vitro* and *in vivo* incubations. The ability to anabolize cholesterol to the C₁₈-C₂₁ steroids that are known to act as bioregulators in vertebrates has been demonstrated in many invertebrate groups and the synthetic pathways have proven to be similar (Sandor & Mehdi, 1979). Such synthetic pathways

Retention Time	% total peak area	3/11/88			
	1	2	3	4	5
33.03	3.39	3.716	2.641	2.854	3.221
34.37	0	0	0.373	0	0
36.80	0.305	0.362	0	0.347	0
38.33	0	0	0	0.09	0
38.70	0	0	0	0	0
39.53	0	0	0	0	0
39.72	0	0	0	0	0
41.25	0.159	0	0	0.138	0
41.90	0	0	0	0	0
43.51	0	0	0	0	0
43.70	0	0	0	0	0
45.50	0.424	0.456	0.793	0.528	0
46.04	4.525	3.409	3.587	4.079	3.832
46.41	0	0	0	0	0
46.95	0	0	0	0	0
47.63	52.119	44.436	48.216	48.504	47.585
47.79	0	0.67	1.079	0.552	1.318
47.88	0.708	0	0	0	0
48.39	0.786	0.643	0	0.628	0
48.71	0.267	0.313	0	0	0.883
49.15	4.474	3.408	3.383	3.833	3.954
49.43	0	0	0	0	0
49.57	0	0	0	0	0
50.08	0	0	0	0	0
51.31	2.434	3.819	2.881	2.185	1.207
51.77	7.591	10.667	10.418	7.561	9.113
52.17	0	0	0	0	0
52.69	0	0	0	0	0
53.13	0.473	0.391	0	0.228	0
53.33	0	0	0	0.289	0
53.75	0.321	0	0	0	0
54.32	0	0	0	0	0
55.32	0	0	0	0	0
55.59	0	0.94	0.737	0.73	0
56.01	1.08	0	0	0	0
56.72	13.583	11.945	10.328	14.279	16.089
56.99	0	0	0	3.412	3.183
58.23	0.838	0.58	0	0.642	0
59.61	0	0	0	0	0
63.88	10.219	14.243	15.563	9.121	9.615

have not been investigated in cnidarians.

The final data chapter discusses the results of sterol profiles taken from extracts of *Favia fragum* over several lunar cycles. The existence of basic sterol molecules and universal precursors such as cholesterol are documented in this chapter. Variability in steroid content over the reproductive cycle is also discussed.

The conclusions and suggestions for building on this work are provided in a final chapter entitled "Future Directions".

Retention time	peak area	3/11/88			
	1	2	3	4	5
33.03	782960	830270	272080	877480	205360
34.37			38430		
36.80	70476	80788		106600	
38.33				27733	
38.70					
39.53					
39.72					
41.25	36734			42301	
41.90					
43.51					
43.70					
45.50	98005	101980	81688	162220	
46.04	1045300	761610	369500	1254300	244340
46.41					
46.95					
47.63	12039000	9928300	4967100	14914000	3034200
47.79		149710	111190	169690	84057
47.88	163530				
48.39	181530	143640		193180	
48.71	61770	70044			56274
49.15	1033500	761430	348520	1178700	252120
49.43					
49.57					
50.08					
51.31	562120	853390	296840	671830	76978
51.77	1753400	2383400	1073200	2324900	581090
52.17					
52.69					
53.13	109200	87436		70063	
53.33				88849	
53.75	74060				
54.32					
55.32					
55.59		210070	75921	224320	
56.01	249500				
56.72	3137500	2668900	1064000	4390400	1025900
56.99				1049200	202970
58.23	193510	129700		197520	
59.61					
63.88	2360500	3182400	1603300	2804500	613110

CHAPTER 2

THE TIMING OF GAMETOGENESIS AND PLANULATION IN THE CORAL, FAVIA FRAGUM, FROM SOUTH FLORIDA

INTRODUCTION

Most scleractinian coral species that have been studied have a periodicity in the release of planula larvae or gametes associated with season and/or lunar phase. The timing of these events is thought to be influenced by several physical factors: annual temperature variation (Harrison et al., 1984; Kojis, 1986), lunar/tidal cycles or variation in nocturnal illumination (Jokiel et al., 1985; Hunter, 1988) and the diel light-dark cycle (Kojis & Quinn, 1981; Babcock, 1984; Miller, 1986). Because these factors vary with latitude, the timing of reproductive events within a species can change with respect to site. Corals nearer to the equator which experience less variation in these parameters are hypothesized to exhibit more continuous reproduction (Oliver et al., 1988). Regional and latitudinal variation in reproductive seasonality, in lunar timing of spawning of gametes or release of planula larvae, and possibly in the mode of reproduction, have been well documented in the Pacific provinces for both broadcasting and brooding species (Richmond & Hunter, 1990 for review). However, in the Caribbean, similar data available from a

Retention time	cholesterol units /mg supernatant protein		3/11/88		
	1	2	3	4	5
33.03	1.464	1.028	0.655	1.368	0.940
34.37	0	0	0.091	0	0
36.80	0.132	0.100	0	0.166	0
38.33	0	0	0	0.043	0
38.70	0	0	0	0	0
39.53	0	0	0	0	0
39.72	0	0	0	0	0
41.25	0.070	0	0	0.065	0
41.90	0	0	0	0	0
43.51	0	0	0	0	0
43.70	0	0	0	0	0
45.50	0.183	0.127	0.195	0.252	0
46.04	1.955	0.943	0.890	1.955	1.117
46.41	0	0	0	0	0
46.95	0	0	0	0	0
47.63	22.517	12.300	11.948	23.242	13.868
47.79	0	0.185	0.268	0.264	0.383
47.88	0.306	0	0	0	0
48.39	0.339	0.178	0	0.301	0
48.71	0.115	0.087	0	0	0.257
49.15	1.932	0.943	0.838	1.837	1.153
49.43	0	0	0	0	0
49.57	0	0	0	0	0
50.08	0	0	0	0	0
51.31	1.052	1.057	0.713	1.047	0.353
51.77	3.280	2.952	2.582	3.623	2.656
52.17	0	0	0	0	0
52.69	0	0	0	0	0
53.13	0.203	0.109	0	0.110	0
53.33	0	0	0	0.138	0
53.75	0.139	0	0	0	0
54.32	0	0	0	0	0
55.32	0	0	0	0	0
55.59	0	0.260	0.183	0.350	0
56.01	0.466	0	0	0	0
56.72	5.868	3.306	2.558	6.843	4.689
56.99	0	0	0	1.635	0.928
58.23	0.362	0.161	0	0.307	0
59.61	0	0	0	0	0
63.88	4.414	3.942	3.857	4.371	2.802

large range in latitude do not show the same trend in change (Wyers, 1985; Szmant, 1986; Soong, 1990; Szmant, 1991; Wyers et al., 1991).

One Caribbean species that has an extensive geographical distribution is the small scleractinian coral, *Favia fragum* (Esper 1795). Its habitat extends across the Caribbean, from the Columbian archipelago San Bernardo and Panama (9°N) in the south (Von Prahl & Erhardt, 1985) to Bermuda (32°N) in the northwestern Atlantic and in the eastern Atlantic in the Cape Verde Islands (Laborel, 1974). The gametogenic, embryogenic and planulation cycles of this brooding species have been described in detail for the Puerto Rican population (Szmant-Froelich et al., 1985), and observations on planulation have been made by several workers in the Caribbean (Table 2.1). In Puerto Rico (18°N), *F. fragum* has a continuous breeding season and exhibits lunar periodicity in spermatogenesis and embryogenesis.

The present study investigates the timing of reproductive events of this species in south Florida (25°N) where the thermal range is much greater than Puerto Rico (Figure 2.1). Greater seasonality in temperature may truncate the reproductive season in south Florida. A histological study of gamete maturation as well as observations of planulation were undertaken on a lunar and seasonal time scale. Planulation observed in other locations in the Caribbean and Atlantic are included to examine variability of periodicity of reproductive

APPENDIX

Table A.1 Gas chromatographic data from Chapter 5.

A.1a Data from 3/11/88

Retention time	cholesterol unit/cm ²	3/11/88			
	1	2	3	4	5
33.03	0.842	0.689	0.215	0.922	0.314
34.37	0	0	0.03	0	0
36.80	0.076	0.067	0	0.112	0
38.33	0	0	0	0.029	0
38.70	0	0	0	0	0
39.53	0	0	0	0	0
39.72	0	0	0	0	0
41.25	0.04	0	0	0.044	0
41.90	0	0	0	0	0
43.51	0	0	0	0	0
43.70	0	0	0	0	0
45.50	0.105	0.085	0.064	0.17	0
46.04	1.124	0.632	0.292	1.318	0.373
46.41	0	0	0	0	0
46.95	0	0	0	0	0
47.63	12.947	8.241	3.919	15.665	4.632
47.79	0	0.124	0.088	0.178	0.128
47.88	0.176	0	0	0	0
48.39	0.195	0.119	0	0.203	0
48.71	0.066	0.058	0	0	0.086
49.15	1.111	0.632	0.275	1.238	0.385
49.43	0	0	0	0	0
49.57	0	0	0	0	0
50.08	0	0	0	0	0
51.31	0.605	0.708	0.234	0.706	0.118
51.77	1.886	1.978	0.847	2.442	0.887
52.17	0	0	0	0	0
52.69	0	0	0	0	0
53.13	0.117	0.073	0	0.074	0
53.33	0	0	0	0.093	0
53.75	0.08	0	0	0	0
54.32	0	0	0	0	0
55.32	0	0	0	0	0
55.59	0	0.174	0.06	0.236	0
56.01	0.268	0	0	0	0
56.72	3.374	2.215	0.839	4.612	1.566
56.99	0	0	0	1.102	0.31
58.23	0.208	0.108	0	0.207	0
59.61	0	0	0	0	0
63.88	2.538	2.641	1.265	2.946	0.936

Table 2.1 Regional comparison of the timing and seasonality of planulation in *Favia fragum* in Caribbean.

Site	Timing of planulation*	Season	Technique	Reference
Panama	< 15 ANM**	all year	dissection	Soong, 1990
Barbados	< 0 ANM > <15 ANM	?	observed	Lewis, 1974 P. Scott cited in Harrison & Wallace (1990)
Jamaica	< 15 ANM *** < 15 ANM	all year	observed	Duerden, 1902 P. Scott cited in Harrison & Wallace (1990)
Puerto Rico	6-12 ANM (peak production)	all year	histology/ observed	Szmant-Froelich et al., 1985
S. Florida Tortugas	< 15 ANM ***	?	observed	Vaughan, 1908 Vaughan, 1910
S. Florida Miami	< 15 ANM	all year	histology/ observed	this study

* ANM = days After New Moon,

** <15 ANM indicates planulation just prior to full moon

*** Lunar phase calculated from literature dates using Meeus, 1983.

MATERIALS AND METHODS

Collection of samples

Twelve to fifteen colonies of *Favia fragum* were collected in an oceanic seagrass bed near Sands Key (25°31'00"N, 80°10'25"W) off the coast of south Florida every ten days for forty days in early October to mid November, 1986. Colonies of reproductive size, greater than 8 polyps and 2.5 cm in diameter, were collected at 1 to 2 meters depth with a hammer and chisel.

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Analysis of gametogenesis and embryogenesis

Histological preparation

Upon return to the Rosenstiel School of Marine and Atmospheric Science, Miami, Fl, corals were placed in outdoor tanks supplied with running seawater and covered with a shading screen. Four colonies were fixed in Zenkers solution (Yevich & Barszcs, 1981) every four days. The samples were decalcified in 10% HCl which contained small amounts of EDTA, sodium tartrate and sodium potassium tartrate. Tissues were processed by standard histological procedures as used by Szmant-Froelich et al. (1985). The embedded tissues were sectioned 7 μ m thick and were stained with Heidenhain's azocarmine-aniline blue counterstain (Luna, 1968).

Microscopic examination

A single section from each of three tissues depths was used to determine the number of each stage of oocyte, spermary, and embryo. The categorization scheme for developmental stages of Szmant-Froelich et al. (1985; see Table 1) was used with the following modification: stage IV and V spermaries were condensed to a single stage representing the mature spermary. All slides were read on an Olympus BH-2 light microscope and magnifications up to 600X were used to make identifications of gametogenic stages.

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Data analysis

The total number of gametes or embryos in each of the four developmental stages was compiled for each date (n=4 colonies per date). The data were subjected to statistical analysis (X^2 - goodness of fit) to compare the number of gonads in each developmental stage across the lunar period at equal intervals beginning with new moon: (1) new moon to first quarter, (2) first quarter to full moon, (3) full moon to third quarter, and (4) third quarter to new moon.

Observation of planulation

Colonies of *Favia fragum* of reproductive size were collected in Biscayne National Park near Alina's Reef (25°23.25'N, 80°09.8'W) on the third day after new moon (3 ANM) on February 18, 1991 (n=8) and on 6 ANM on March 23, 1991 (n=9). Colonies were placed in outdoor planulation chambers designed to catch planulae from individual colonies (Szmant-Froelich et al., 1985). The chambers were inspected for planulae every morning from 3 ANM to 18 ANM in February and from 7 ANM to 19 ANM in March. Additional observations on planula release were made on freshly collected colonies in the laboratory at irregular intervals.

RESULTS

Reproductive activity

All of the 41 colonies examined histologically contained gametes. Eighty percent of all colonies collected were

420-423.

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brooding. This result agrees with that found in Panama for colonies collected across the lunar series (70% expected to be brooding, Soong, 1990). Four of the eight colonies which did not contain planulae were those collected 9 to 12 ANM presumably following a planulation event.

Gametogenesis and embryogenesis

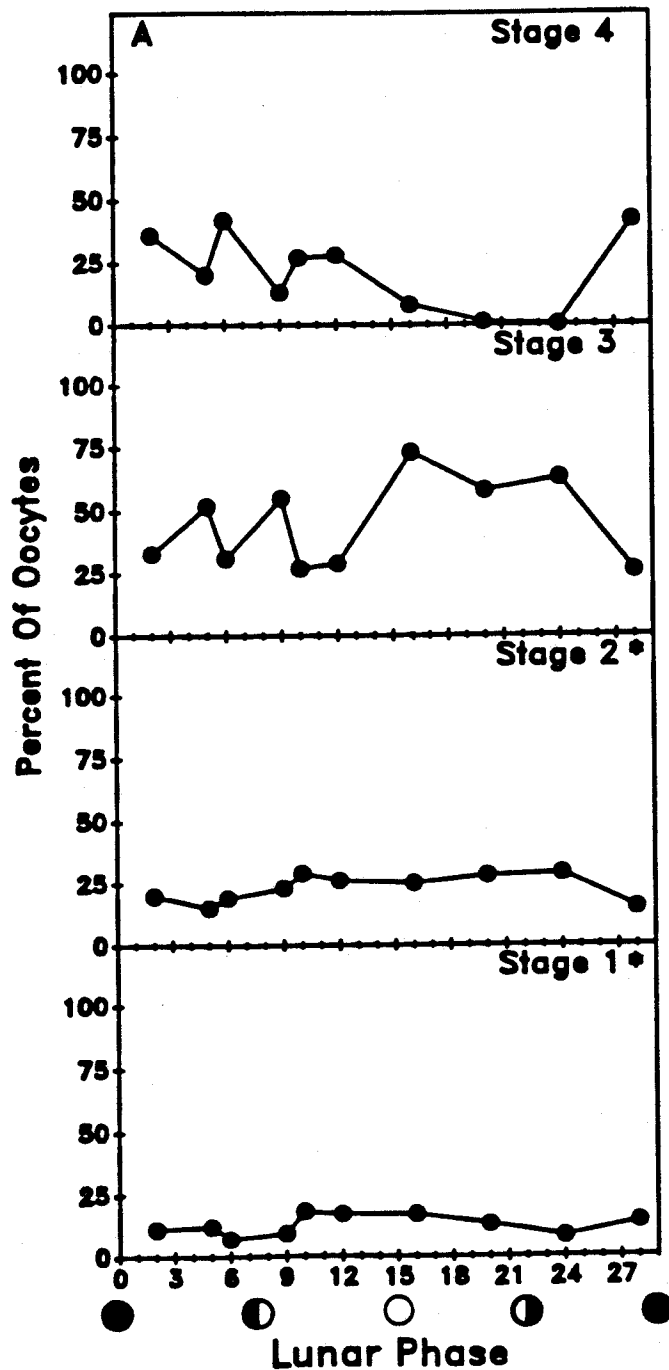
The temporal distribution patterns of oocyte, spermary and embryonic stages are illustrated in Figure 2.2 a,b, and c. Cycles of gametogenesis and embryogenesis of *Favia fragum* are in agreement with those of Szmant-Froelich et al. (1985) for Puerto Rico. The lunar timing of the appearance of maturing gametes (Stage III and IV) in the histological sections concurs with the timing of appearance of larger gametes found by dissection of live colonies from Panama (Soong, 1990).

The proportion of stage III and IV oocytes changed significantly over the lunar cycle (Figure 2.2a, χ^2 , $p < 0.005$) while early stage oocytes maintain a constant proportion of the oocytes throughout the lunar cycle. This suggests a lunar periodicity in the maturation of oocytes but not in the onset of oogenesis. Ripe oocytes (Stage IV) are most numerous in the first lunar phase (0-9 ANM) prior to fertilization.

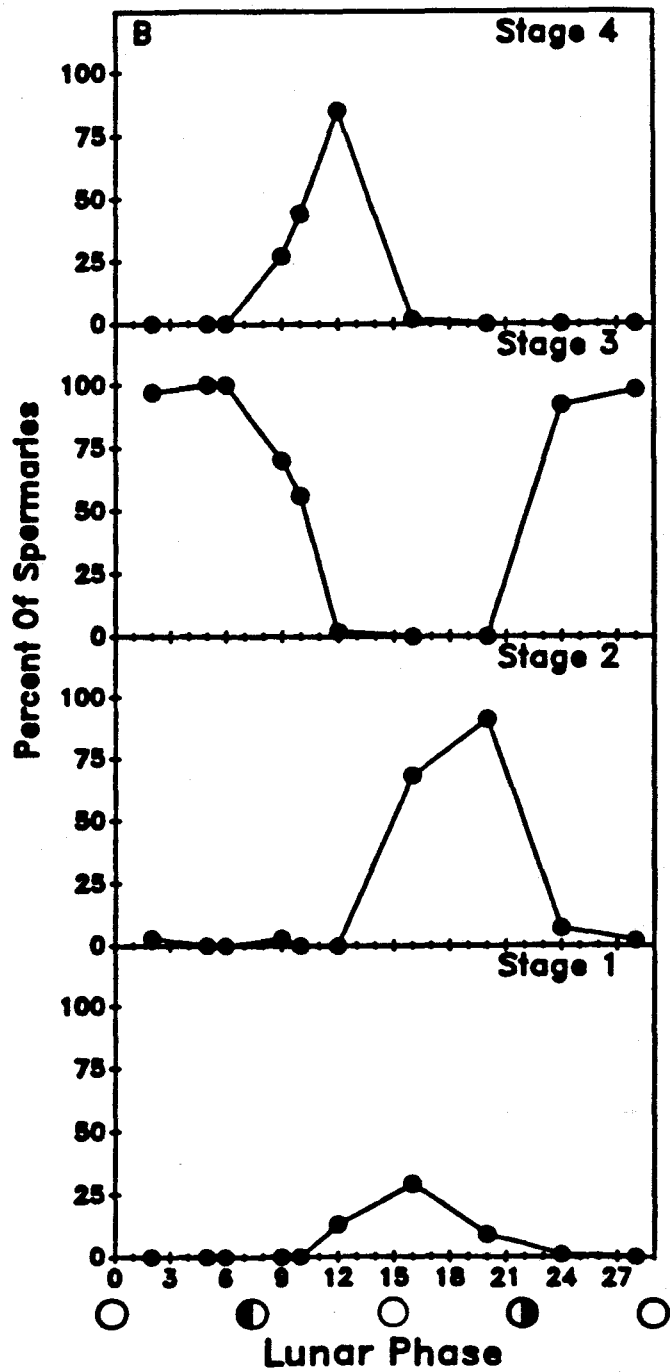
Spermaries and embryos show a complete progression of developmental stages within one lunar cycle (Figure 2.2b and 2.2c, χ^2 , $p < 0.005$), demonstrating lunar periodicity in appearance and development of male gonads and larvae.

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Figure 2.2A-C. Summary of the reproductive cycles of *Favia fragum*. Gametogenic and embryogenic cycles of *F. fragum* from south Florida were determined by examination of histological samples. Each graph represents the percent of total gametes or embryos assigned to a given stage for each collection day (n=4 colonies). The distribution of the number of gametes or embryos within a stage over the four lunar periods was significantly different except were noted by * (Fig. 2.2A, $X^2 > 35.2$; Fig. 2.2B, $X^2 > 124$; Fig. 2.2C, $X^2 > 140$; $p < 0.005$, d.f.=3).



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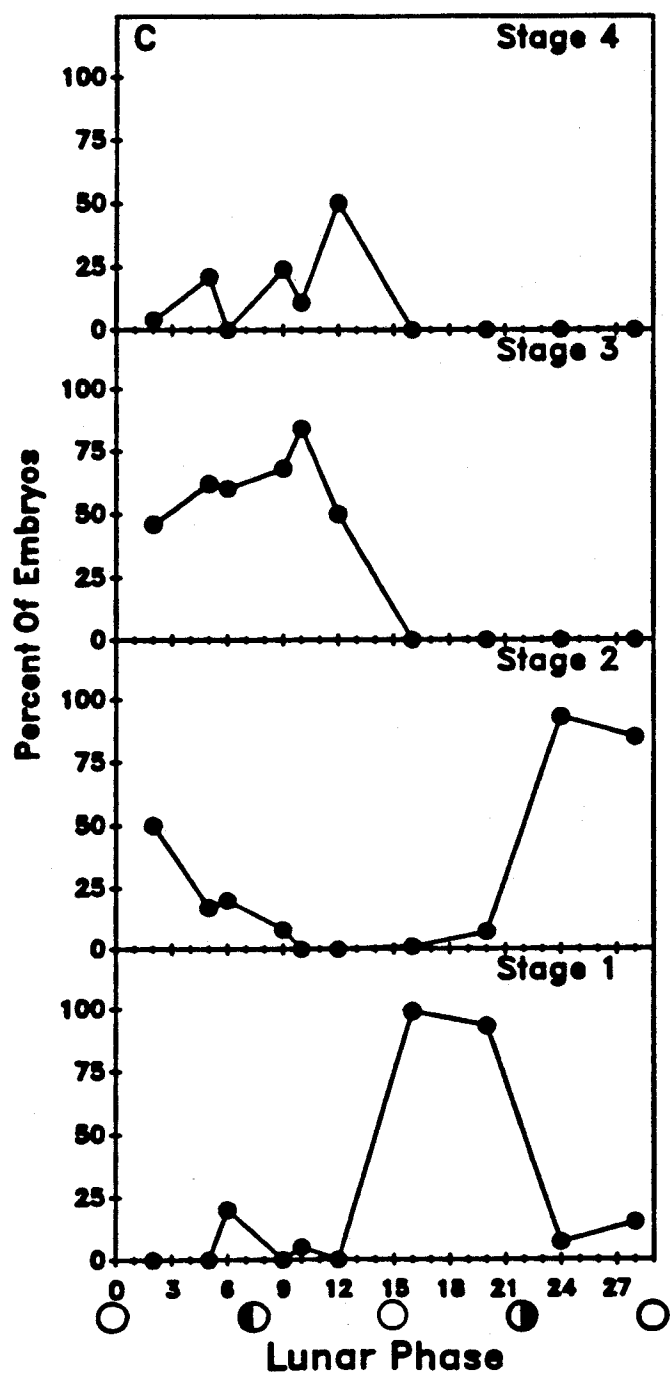
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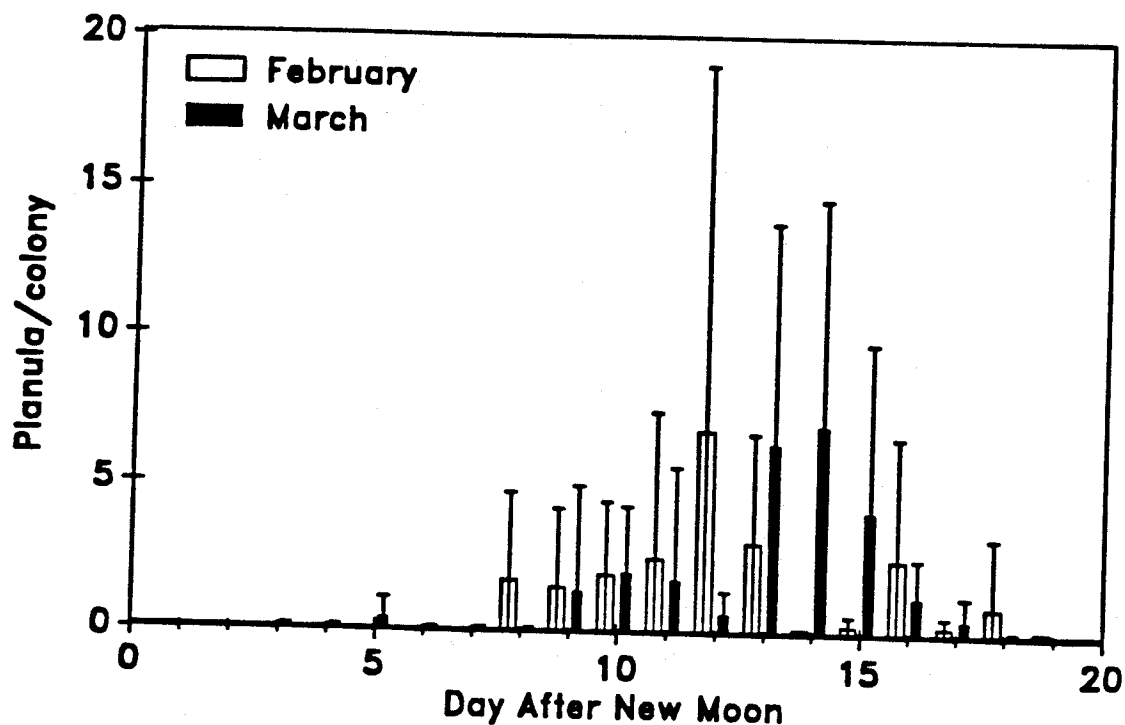
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Figure 2.3 Planulae released per colony of *Favia fragum* (February, 1991 n=8; March, 1991 n=9). Bars are mean number of planulae released per colony with standard deviation given. Peak planula release occurred a few days full moon. Planula release continued to occur several days after the full moon.



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Planulation

The disappearance of mature planulae from histological sections occurs before full moon in the south Florida samples (Figure 2.2c). Freshly collected colonies were noted to planulate in the laboratory in November 1989 (day 8 ANM), July 1990 (day 6 ANM), February 1991 (day 5-15 ANM), and March 1991 (day 7-18 ANM) (Figure 2.3). Planulation observed in the summer, fall, and winter provides evidence that *Favia fragum* reproduces continuously throughout the year. The peak in planula release occurred from day 12-15 ANM. This is several days later than peak planulation observed in Puerto Rico (Szmant-Froelich et al., 1985).

DISCUSSION

Lunar timing of planulation

Planulation by the coral, *Favia fragum*, has been observed and recorded by workers in sites throughout the Caribbean (Table 2.1). Planula release occurs prior to the full moon across the entire latitudinal gradient from Panama (9°N) to Miami, FL (25°N) with the exception of Barbados from which there are conflicting reports. The conservation of the timing of planulation across latitudinal lines indicates either that temperature and variation in the diel light/dark cycle does not affect mechanisms responsible for larval release or that this degree of temperature and light variation is insufficient to cause a change in the release of planulae. The peak in

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planula release from *F. fragum* in S. Florida is delayed by several days when compared to planulation in this species from Puerto Rico (Szmant-Froelich et al., 1985). Since the timing of the onset of gametogenesis and spawning are the same in both populations, the corals from the higher latitude may be brooding longer. The planulae may require more time to reach maturity when the coral animal experiences cooler temperatures or metabolic limitations due to shorter photoperiods.

Periodicity and seasonality of gametogenic events

The timing and progression of gametogenic events in *Favia fragum* colonies from S. Florida (Figure 2.2) do not vary from those found in Puerto Rico (Szmant-Froelich et al., 1985) and Panama (Soong, 1990). The reproductive cycle is continuous over the year despite increasing variation in photoperiod and thermal range at higher latitude (Figure 2.1). In this case, latitudinal variation in temperature fails to produce a change in the annual breeding periodicity of *F. fragum* predicted for the breeding of corals and other marine animals (Orton, 1920; Yonge, 1940; Giese & Pearse, 1974). Van Moorsel (1983) hypothesizes that colonies which are small as adults and living in unstable shallow environments are more likely to breed continuously regardless of annual variation in other parameters. Colonies of *F. fragum* fall into this category and therefore would be expected to release planulae year round across all latitudes where it is found.

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Although the reproductive periodicity of *Favia fragum* did not vary among sites, other characteristics may differ between higher and lower latitudes. Differences in environmental condition between populations may affect the reproductive output as measured by number of planulae, egg volume or number. For example, the number of spawning events and size of reproductive products vary along a latitudinal gradient on the Great Barrier Reef for *Acropora palifera* (Kojis, 1986). The number of planulae released by colonies of *Pocillopora damicornis* can be maximized at optimal temperature (Jokiel & Guinther, 1978). In *Agaricia humilus* and *Porites astreoides*, there is a seasonal peak in planula production (Van Moorsel, 1983; Chornesky & Peters, 1987). However, in *F. fragum*, the number of planulae released varies between colonies and between reefs in the same geographical and environmental location (A. Szmant, personal communication; K. Cole, unpublished data). This parameter could be used as a measure for reproductive output if large sample sizes were used to compensate for the variability.

Annual variations in temperature and light may have a greater effect on the length of the reproductive season for broadcasting species which spawn during one or two brief periods each year. Spawning season of three Caribbean broadcasting species in the family Faviidae, *Montastrea annularis*, *Montastrea cavernosa*, and *Diploria strigosa*, is consistent from low to high latitude (Wyers, 1985;

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Szmant, 1986; Soong, 1990; Szmant, 1991; Wyers et al., 1991) occurring July through September. However, the length of the gametogenic season, from onset to spawning, has not been reported at all three sites nor has there been a comparison of relative reproductive output.

Caribbean versus Pacific

The timing of reproductive events in 47 Pacific coral species have been shown to vary with latitude and geographical location (Richmond & Hunter, 1990). However, on the Great Barrier Reef, synchronized reproductive events within populations, such as mass spawning, have been observed (Babcock et al., 1986; Harrison et al., 1984). Variation in reproductive timing within species and synchronization between species has not yet been demonstrated in the Caribbean. This may be due in part to a steeper gradient of environmental conditions in the Pacific. Another factor may be that the reproductive characteristics of two thirds of the Caribbean species have yet to be investigated and few studies (Soong, 1990; this study) have looked at variation between sites. Soong (1990) has suggested that gene flow among regions in the Caribbean maintains homogeneity of reproductive characteristics. For this reason, it would be interesting to document the reproductive cycle for *Favia fragum* in the Cape Verde Islands, considering its potential for reproductive isolation from the rest of the species. Studies of the

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reproductive characteristic of *F. fragum* from Bermuda (Wyers, unpublished data) may answer additional questions of seasonality for the western Atlantic population.

In conclusion, the lunar and seasonal timing of gametogenic events in the south Florida population of *Favia fragum* was the same as those reported for other areas of the Caribbean. However, it appears that planula production is less than that observed in Puerto Rico and that the planulae are brooded longer. Other reproductive characteristics, such as egg volume or number of planulae released per month, may have to be investigated to show differences between populations of Caribbean coral species which fall along a latitudinal gradient.

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CHAPTER 3

CYTOCHROME P-450 CONTENT AND XENOBIOTIC METABOLIZING ENZYME ACTIVITIES IN THE SCLERACTINIAN CORAL, FAVIA FRAGUM (ESPER)

INTRODUCTION

The effect of anthropogenic inputs of xenobiotic compounds such as pesticides and petroleum products on the fragile marine environments of coral reefs has been the subject of growing concern. Field studies following contamination events such as oil spills have documented hard-coral mortality, shifts in community structure, and a slow recovery in these areas (Loya & Rinkevich, 1980; Jackson et al., 1989). Long-term monitoring programs have shown that corals in polluted areas suffer from sublethal effects such as a reduction in reproductive capacity (Rinkevich & Loya, 1977) and changes in lipid metabolism (Burns & Knap, 1989). Laboratory experiments on shorter time scales have shown similar sublethal effects to corals in the presence of petroleum products (Rinkevich & Loya, 1979; Peters et al., 1981).

The uptake of lipid-soluble xenobiotics by corals is rapid (Knap et al., 1982; Solbakken et al., 1984), while the elimination of such compounds is slow compared to rates found

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in other marine invertebrates (Solbakken et al., 1982, 1983, 1985). For example, in the tissues of the scleractinian coral, *Diploria strigosa*, 84% of the administered radioactivity was still present one year after exposure to radiolabeled 2,4,5,2',4',5'-hexachlorobiphenyl (PCB) (Solbakken et al., 1984). However, the proportion of radioactivity as parent PCB or PCB metabolite was not determined. The presence of organochloride pesticides (Glynn et al., 1989), herbicides (Glynn et al., 1984), and petroleum hydrocarbons (Burns & Knap, 1989; Jackson et al., 1989) in coral tissue from polluted areas as well as the long half-lives of many of these compounds suggests that the rates of xenobiotic detoxification or elimination may be especially low.

The capacity of an organism to metabolize xenobiotics has substantial effects on the tissue levels of toxicants, metabolite patterns and xenobiotic half-life in an organism, all of which may affect the severity and duration of a toxic response (Buhler & Williams, 1989). The metabolism of many xenobiotics consists of several sequential enzyme-catalyzed reactions. The cytochrome P-450-dependent mixed-function oxidase (MFO) system is responsible for the oxidation of many endogenous compounds (e.g., steroids, vitamins, prostaglandins, and fatty acids), but it is also responsible for catalyzing the initial oxidation (i.e., Phase I reaction) of exogenous compounds (e.g., organochloride pesticides and

fragum will undoubtedly provide additional answers to the question of which compounds cycle over the month, the answer to the question of whether these compounds have bioregulatory function may be elusive. Compound isolation and structural identification will be an important step preceding careful experimentation to discern physiological function of specific compounds. The use of blocking agents specific for certain enzymes in the steroid pathways may be useful in isolating which chemical products are important in reproduction.

Natural products chemists have become interested in the chemical aspects of coral reproduction following the mass spawning event. Especially in the soft corals, several compounds, thunburgol (Bowden et al., 1988), pukalide, and epoxypukalide (Coll et al., 1989) are now associated with gametes and gamete production. Terpene compounds which cause polyp muscle contractions have also been found (Pass et al., 1989). The study of such terpenes in seasonally reproducing corals may be the best direction to take in understanding which chemicals which influence reproduction of scleractinian corals in the Caribbean.

polycyclic aromatic hydrocarbons) in the detoxification process (Black & Coon, 1987). Subsequent reactions (i.e. Phase II) involve the conjugation of the original compound or its Phase I metabolites to an endogenous hydrophilic molecule such as glucuronic acid. Thus, Phase I and II enzymes typically facilitate the excretion of xenobiotics by increasing their hydrophilicity.

Cytochrome P-450 and many xenobiotic metabolizing enzymes are inducible, i.e. increased levels and different isozymes are often found in organisms which have been exposed to various pollutants (Buhler & Williams, 1989). Native levels of such enzymes may be below detection limits and may reach levels which are measurable only after induction.

As mentioned earlier, the MFO system and at least one Phase II enzyme (UDP-glucuronosyltransferase) function endogenously in the biosynthesis and metabolism of steroid hormones. In fish, they have been shown to change significantly with season, corresponding to the reproductive cycle of the species (Koivusaari, 1983; Andersson & Koivusaari, 1985). Seasonal changes in the level of aryl hydrocarbon hydroxylase activity have also been measured in crabs (Lee et al., 1982). MFOs have been found in many marine invertebrates including echinoderms (Den Besten & Livingstone, 1989), arthropods, annelids and molluscs (Lee, 1981) but have not been reported in marine cnidarians such as sea anemones (Payne, 1977; Lee, 1981), jellyfish and scleractinian corals.

has been addressed but what is their functional role?

In addition to steroid metabolism, MFO enzymes systems play a role in xenobiotic metabolism of polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and other pollutants. Their function as detoxifying enzymes justifies continued investigation into their characteristics in these lower invertebrates. Any further investigations should be focussed first on optimizing buffer and assay conditions for cnidarians. (Current methods are usually described for piscine, molluscan and crustacean species.) Laboratory experiments are necessary to adequately demonstrate inducibility of MFOs in corals as well as to characterize the types of compounds (PAH or PCB) that increase enzymatic activity.

One of these enzymes, UDP glucuronosyltransferase, has not been found in invertebrates using glucuronic acid as a conjugation substrate (Dutton & Burchell, 1977; Schell & James, 1989). Most invertebrates use glucose as a substrate. A comparison of the enzymatic activity using the two conjugation substrates is necessary to substantiate the claim the corals are performing an enzymatic function which suggests a significant departure from other invertebrate phylogenetic groups.

Chemicals important to reproduction

While careful analysis of the sterol profiles of *Favia*

The objectives of this study were to establish baseline levels of cytochrome P-450 content and the activities of four xenobiotic metabolizing enzymes, to look for evidence of prior induction and to investigate the temporal variability of enzyme activities, in the scleractinian coral, *Favia fragum*. In order to examine induction in *F. fragum*, a comparison of the activities of several xenobiotic metabolizing enzymes from corals which may have been exposed to known inducers of the MFO system in coral colonies collected from three sites, in which the offshore areas were unpolluted relative to the inshore areas (P. Glynn, personal communication). This caribbean coral is a hermaphrodite which completes spermatogenesis, matures a limited number of oocytes, and broods and releases planula larvae within a single lunar cycle (Szmant-Froelich et al., 1985). Therefore, in order to search for temporal differences in cytochrome P-450 content and several xenobiotic metabolizing enzyme activities which may be associated with the regulation of reproduction, several colonies from one site were collected over the lunar cycle (the cycle of gametogenesis).

MATERIALS AND METHODS

Sampling Sites and Collection Methods

Collection size was restricted according to permits issued by state and federal agencies. Three colonies of *Favia fragum* were collected from each of three sites in Key Largo

period revealed no patterns of increased activity with different gametogenic events in *Favia fragum*.

Little background was available on the steroidogenic capabilities of cnidarians. *Favia fragum* was able to metabolize the steroid, progesterone, in both *in vitro* and *in vivo* incubations, into a large number of metabolites including other vertebrate steroids (Chapter 4). MFO enzymatic systems most likely contributed to these biotransformations (Chapter 3). Some authors suggest that the ability to transform one compound into another indicates physiological function of those metabolized compounds (Swevers et al., 1991). However, without knowledge of the presence or levels of such steroids within the coral tissues, we can not begin to predict the physiological function of such enzyme systems. Radio-immunoassay would be the best choice of method to confirm the presence of androgen and estrogen type compounds in corals. It should be noted that other cnidarians make C₂₁ steroids (Higgs & Faulkner, 1977) and that several compounds elute in the range of C₁₉ and C₂₁ steroids on GC (Chapter 5). While the discovery of vertebrate-type prostaglandins in gorgonians (Weinheimer & Spraggins, 1969) encouraged investigation into gorgonian cyclo-oxygenases and lipoxygenases (Morse et al., 1978; Doerge & Corbett, 1982; Bundy et al., 1986; Brash et al., 1987), no such interest has been sparked for the biochemistry of steroids in corals. The basic question of whether these steroid metabolizing systems operate in corals

Marine Sanctuary (KLMS), Florida: Kemphouse Reef (KR) (25°13.80'N, 80°19.50'W, inshore), a patch reef in Basin Hills Shoal (BH) (25°12.80'N, 80°14.60'W, midshore), and south Carysfort Reef (SC) (25°12.70'N, 80°13.00'W, offshore) in November, 1989 (over a three day period corresponding to lunar day 3-5 after new moon (ANM) and processed on day 8 ANM). The inshore site (KR) was contaminated with polyaromatic hydrocarbons which are known inducers of the MFO system. Preliminary analyses of concurrently collected sediment samples have shown hydrocarbon concentrations to be 19 times greater at the inshore site than the most offshore site (SC). Several polycyclic aromatic hydrocarbons (PAHs) were detected. The concentrations of these PAHs at the inshore site were three to sixty-five times greater than the offshore sites (P. Glynn, University of Miami, unpublished data).

Two colonies for the lunar series were collected every 3 to 5 day from October to mid November, 1989 from several patch reefs near Alina's Reef (25°23.25'N, 80°09.8'W) in Biscayne National Park (BNP), Florida. Whole colonies (2 to 5 cm in diameter) were dislodged from the substrate with hammer and chisel. Corals were transported to the laboratory in pinch-sealed plastic bags with 250-300 ml of seawater at ambient temperature.

Enzyme Preparation

Microsomes were prepared immediately upon return to the

seasonal peak in planula output (or reproductive effort).

Planula release may be^e delayed in Florida because it takes longer for embryos to reach maturation, possibly due to latitudinal difference in temperature range. The effect of lower temperatures on length of brooding could be tested in the laboratory using temperature controlled aquaria exposed to natural light. A comparison of reproductive patterns of *Favia fragum* in south Florida to those in Bermuda which experience lower temperatures and those in the Cape Verde Islands would test the effects of latitudinal differences.

In broadening the view to include all coral species in the Caribbean, one discovers that the reproductive characteristics of only 21 coral species are known. Basic research on gender distribution, seasonality of breeding and mode of reproduction await the other 70% of the Caribbean species. Questions concerning the energetics of reproduction, biochemical and environmental regulation require thorough investigation to advance our understanding of this complex reef ecosystem.

Steroid metabolizing enzymes

The experiments described in Chapter 3 provided baseline data on the activities of mixed function oxidase (MFO) enzymes previously undetected in cnidarians (Payne, 1977; Lee, 1981). Higher activity of some enzymes were seen in inshore sites known to have higher concentrations of pollutants, suggesting induction of the enzyme systems. A time course over one lunar

laboratory (except for KLMS samples) as follows: all tissues were removed from the coral skeleton of each colony with a jet of high pressure (70 psi) air and homogenization buffer (0.15 M KCl, 0.02 M N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH 7.4) from an artist's airbrush. The surface area of each coral colony was later determined by the aluminum foil method of Marsh (1970). The tissue was homogenized on ice for 30 s with an Ultra-Turrax homogenizer which disrupted the animal tissue but left the zooxanthellae intact. The homogenates were then centrifuged at 2000 g for 4 min at 4°C in a Sorval RC5B superspeed centrifuge to remove zooxanthellae and skeletal elements. The supernatant containing coral tissue was decanted and homogenized at 4°C with a Potter-Elvehjem glass-teflon homogenizer in 4 volumes of ice-cold homogenization buffer. The homogenates from coral tissue were centrifuged at 10,000 g for 20 min in a Sorvall RC5B superspeed centrifuge. The resulting supernatant was centrifuged at 100,000 g for 1 hour at 4°C in a Model L7 Beckman ultracentrifuge. The 100,000 g supernatant (cytosol) was removed and the surface of the microsomal pellet was rinsed gently with 1 ml of the homogenization buffer. Microsomes were resuspended in homogenization buffer plus 20% glycerol (v/v). Microsomes and supernatant were stored for no longer than 80 days at -80°C prior to analysis.

CHAPTER 6

FUTURE DIRECTIONS

The objectives of this study were to document the reproductive cycle of the scleractinian coral *Favia fragum* in south Florida, explore aspects of its steroid biochemistry and look for cycling of sterol compounds in its tissues over the lunar period which could be correlated with reproductive events. As in all research, some questions were answered suggesting new questions to be asked. This concluding section summarizes the results of this dissertation and points the way for future research projects.

Reproduction in *Favia fragum*

Histologically, the gametogenic development of *Favia fragum* in south Florida (Chapter 2) doesn't differ from populations in Puerto Rico (Szmant-Froelich et al., 1985). However, the number of planulae released is fewer than that reported by other studies. The planulae appear to be brooded for longer periods of time. More frequent observation of planulation over an annual cycle may reveal that *F. fragum* resembles *Porites astreoides* (Chornesky & Peters, 1987) and *Agaricia humilis* (van Moorsel, 1983) in having continuous gametogenesis but a

Enzyme Activities

Microsomal and cytosolic (soluble) protein concentrations were measured by the method of Lowry et al. (1951), with bovine serum albumin (Sigma) as a standard.

Cytochrome P-450 content was determined by the method as described for the polychaete *Nereis virens* by Fries and Lee (1984) according to Omura and Sato (1964a), which measures the CO-difference spectra of dithionite-reduced microsomes. A spectral profile of dithionite-reduced microsomes from a fresh sample prepared as above was measured from 400-500 nm on a Response II UV-VIS spectrophotometer (Gilford Systems, Oberlin, OH).

Benzo[a]pyrene hydroxylase (BaPH) activity was determined by the modified radiometric procedure of Van Cantford et al. (1977) as described by Collier et al. (1986). A mixture of 450 μ l 50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM NADPH and 37.5 μ l microsomal suspension was pre-incubated for 5 min at room temperature (23°C). The addition of 12.5 μ l acetone containing 40 nmoles (2 μ Ci) ^{14}C -benzo[a]pyrene (BaP) started the reaction. In control flasks, KOH:DMSO was added before BaP. Incubations were for 15 min at room temperature and the reaction was stopped by the addition of 1 ml of 0.15 M KOH in 85:15 (v/v) DMSO:H₂O. The reaction mixture was extracted three times with 5 ml hexane to remove the unreacted BaP. The aqueous layer was neutralized with 0.5 ml 0.1 N HCl and 0.5 ml removed and analyzed by liquid scintillation counting (LSC).

temperatures prevented taking full advantage of the splitless injection technique. Increased resolution provided by the narrowing of peak widths may facilitate the use of shorter columns (e.g. 30 m instead of 60 m) which would reduce the run time per sample.

CONCLUSIONS

The technical problems which plagued this aspect of the research project can be overcome. While the present results did not provide answers, they did provide direction. The 1988 sampling results indicate differences in the overall sterol profile over the lunar period of reproduction in *Favia fragum*. Future attempts at this type of sampling may be better applied to seasonally reproducing species in which the gametogenic events are more punctuated. Results may be more readily interpreted and correlated to the specific gametogenic events.

Epoxide hydrolase (EH) activity was assayed by the modified radiometric procedure of DePierre et al. (1975) according to Collier et al. (1986). Reaction vials contained 75 μ l 0.5 M Tris/HCl, pH 7.5, and 25 μ l microsomal suspension. A reaction was started by the addition of 40 μ l acetone containing 0.58 μ moles (3.6 μ Ci) of ^{3} H-styrene oxide (Amersham) and was continued for 30 min at room temperature.

In the control vial, hexane was added before ^{3} H-styrene. The reaction was stopped by the addition of 5 ml hexane. Water (1.9 ml) was added to the vial and the organic phase discarded. The aqueous layer was extracted three times with 5 ml hexane to remove any remaining substrate. A 1 ml aliquot of the aqueous phase was removed and analyzed by LSC.

UDP-glucuronosyltransferase (UDPGT) activity was measured by a modified spectrophotometric method of Hellman and Touster (1962). The reaction mixture, in a total volume of 0.6 ml, contained 60 μ l of 1.7 mM p-nitrophenol in 0.5 M $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 7.4, 100 μ l of 0.1 mM UDP-glucuronic acid, 50 μ l of microsomal suspension, 140 μ l H_2O and 250 μ l 0.1% Triton X-100 in 0.5 M $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$. Control vials contained no UDP-glucuronic acid. The reaction was continued for 30 min and was stopped by the addition of 2.4 ml of 0.1 N trichloroacetic acid (TCA). The mixture was centrifuged at 2000 g for 10 min and 60 μ l of 10 N KOH added to the supernatant. The absorbance was measured at 401 nm.

Glutathione-S-transferase (GST) in cytosol was measured

variations in FID response and not a derivatization artifact.

Controls

The experimental procedure used had several barriers to quantitative analysis. First, the airbrush method fails to remove all the tissue present in the coral colony. Extraction of the whole colony was avoided so as to isolate the animal tissue fraction away from the zooxanthellae and endolithic organisms. Normalization of the resulting chromatographic data to surface area may only reflect the efficiency of the air brush technique and not individual sample variation. The addition of a known compound just prior to extraction (e.g. one of the artificial estrogens like ethinyl estradiol; Fig. 5.7), would provide a control of the extraction and separation efficiencies. This efficiency could be used to calculate absolute amounts of each compound.

Gas chromatographic analysis

The use of splitless injection allows the majority of the injection to be placed on the column. The initially vaporized sample reconcentrates at the head of the column. Splitless injection coupled with a decrease in initial column temperature below the boiling point of the carrier solvent (hexane, b.p. 69°C), would provide better separation by narrowing the peak width. Our use of higher initial column

spectrophotometrically as described by Habig et al. (1974). The reaction mixture consisted of 1.95 ml of 50 mM HEPES, pH 7.5, 40 μ l of 1-chloro-2,4-dinitrobenzene and 25 μ l of 100,000 g supernatant. The reaction was started by adding 25 μ l of 50 mM reduced glutathione in 50 mM HEPES. Controls were performed both without supernatant or glutathione in the reaction mixture. The change in absorbance at 340 nm was monitored for 1-2 min.

A LKB Biochrom Ultrospec 4050 spectrophotometer was used for spectral measurements. Radioactivity was counted on a Tracor beta liquid scintillation counter. ACS (Amersham) was used as scintillation liquid in LSC. All biochemicals were purchased from Sigma (St. Louis).

Calculations and statistics

Enzyme activities were normalized to microsomal or soluble protein. After testing for homogeneity of variance, all data were compared using the Kruskal-Wallis one-way analysis of variance (Zar, 1974) to test for differences between corals from different locations on a protein or surface area basis. A statistical comparison of data collected over the lunar cycle was not possible because of small sample size due to limitations on coral collection. Due to the low levels of cytochrome P-450 and several enzyme activities, a minimum detection limit of two times greater than control values was designated.

tubes, some degradation inevitably occurred. A better approach, regardless of the anticipated storage time, would have been to store in 2:1 CHCl_3 :MeOH using an antioxidant such as tocopherol or butylated hydroxytoluene at 0.05% (Kates, 1986) and storing at a lower temperature (-40 to -70°C).

Derivatization

The internal standard, nortestosterone, formed two peaks. This was due to one of two factors; incomplete derivatization of the parent molecule or formation of keto-enol TMS derivatives. The first explanation is unlikely because other standards derivatized in the same manner did not form dual peaks. Under the derivatization conditions, the alcohol at C-17 should have taken on a trimethylsilyl group (TMS). However, the ketone at C-3 may also have been derivatized as an enol-TMS. Milder experimental conditions, e.g. derivatization at room temperature, may have prevented the derivatization of the carbonyl at C-3. The formation of the enol, especially in the presence of air, may have caused this secondary peak.

A better choice as an internal standard would be a compound that would not require derivatization such as ergostane, etiocholane or benzopyrene (Figure 5.7). The standard should then be used to make a stock solution of known concentration used to re-solvate samples following derivatization but prior to injection into the GC. The standard, treated in this manner, would provide a true internal control, reflecting only

RESULTS

Cytochrome P-450 was found in most *Favia fragum* samples (0.09 ± 0.16 pmol/mg microsomal protein, $n=25$) and a typical carbon monoxide difference spectrum showing the presence of cytochrome P-450 in the microsomes of *F. fragum* is shown in Figure 3.1.

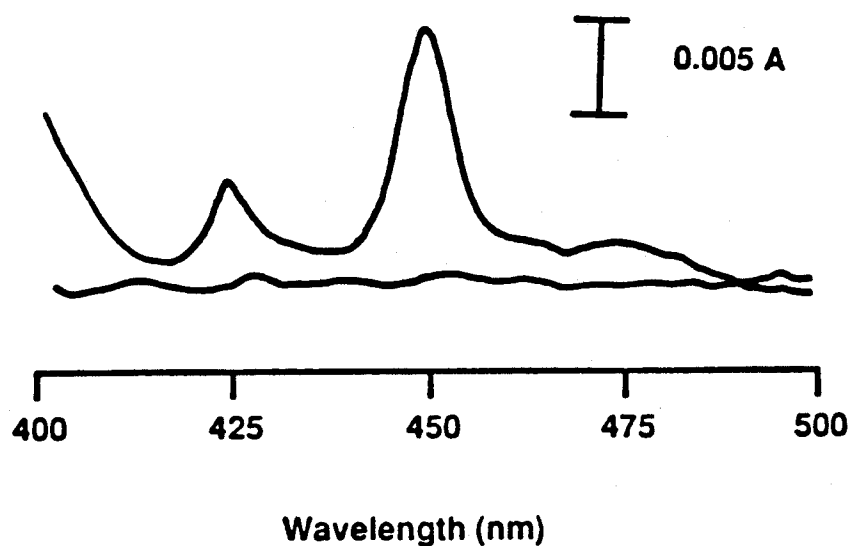


Figure 3.1 A typical carbon monoxide difference spectrum of dithionite-reduced microsomes from *Favia fragum*.

Lipid gravimetrics

Total lipid were weighed to one tenth of a milligram on a four decimal place gram analytical balance. Much higher precision and accuracy could have been achieved by using a microbalance and desiccated samples. Alternatively, to prevent contamination of the sample, the use of a spectrophotometric assay for total lipid such as the phosphosulphovanillin method (Barnes & Blackstock, 1973) could have been performed on an aliquot of the total lipid. Better lipid weights could have been used to assess the relative contribution of the sterols to the total lipids.

Sample storage

The 1988 samples were collected, processed and analyzed within a one month time period. Sample integrity was never compromised. However, in the 1989 lunar sampling, colonies were collected over a two month period. While extractions were performed on fresh samples, the lipid residues were kept frozen for up to three months before undergoing the first set of column chromatography. Final gas chromatographic analysis occurred nearly one year later due in part to the number of samples which needed to be analyzed and technical difficulties including the malfunction of the GC thermistor.

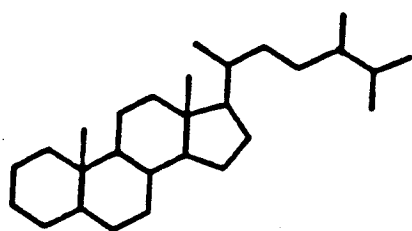
There is a general lability of lipids toward peroxidation and hydrolysis in storage. While these samples were initially stored dry at -4 to -20°C under nitrogen in tightly capped

Table 3.1 Enzyme activities normalized to mg protein and compared by collection site. Benzo[a]pyrene hydroxylase (BaPH), epoxide hydrolase (EH), UDP-glucuronosyltransferase (UDPGT), glutathione-S-transferase (GST), cytochrome P-450, microsomal protein and soluble protein content in the isolated microsomes and cytosol of *Favia fragum* were collected from three sites in Key Largo Marine Sanctuary: Kemphouse Reef (KR), Basin Hills Shoal (BH) and South Carysfort Reef (SC). Values are means $\pm$ S.E. of individual coral colonies (n=3 per site) and an * denotes a significant difference in activities or content at $p < 0.06$ level among sites (Kruskal-Wallis one way analysis of variance).

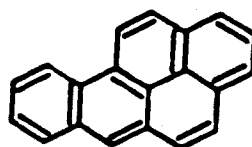
Enzyme	Location		
	KR	BH	SC
BaPH* (pmol/min/mg)	3.78 $\pm$ 1.40	1.15 $\pm$ 0.41	0.95 $\pm$ 0.10
E H (nmol/min/mg)	9.66 $\pm$ 1.56	13.5 $\pm$ 0.80	8.77 $\pm$ 1.18
GST (nmol/min/mg)	18.7 $\pm$ 7.6	16.0 $\pm$ 6.8	10.4 $\pm$ 7.4
UDPGT* (pmol/min/mg)	15.3 $\pm$ 1.56	4.21 $\pm$ 0.58	4.10 $\pm$ 0.39
Cytochrome P-450 (nmol/mg)	0.06 $\pm$ 0.04	0.12 $\pm$ 0.02	0.06 $\pm$ 0.05
Microsomal protein (mg/cm ²)	0.35 $\pm$ 0.07	0.22 $\pm$ 0.06	0.25 $\pm$ 0.03
Soluble protein (mg/cm ²)	0.42 $\pm$ 0.05	0.31 $\pm$ 0.04	0.28 $\pm$ 0.02

Figure 5.7 Compounds proposed for use as analytical standards.

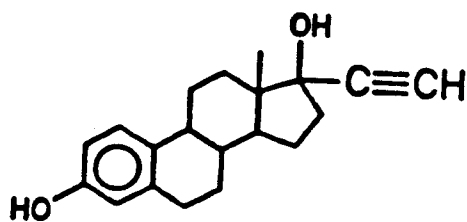
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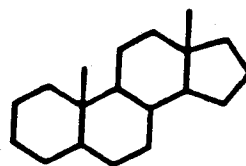
Ergostane



Benzopyrene



Ethinyl Estradiol



Etiocholane

Xenobiotic metabolizing enzyme activities (normalized to microsomal protein except where noted) were also found in most of the corals sampled (BaPH, 1.26 ± 0.97 pmol/min/mg; EH, 14.1 ± 6.9 nmol/min/mg; UDPGT, 2.73 ± 1.72 pmol/min/mg; GST, 15.2 ± 7.8 nmol/min/mg soluble protein, $n=25$). No significant differences in cytochrome P-450, microsomal or soluble protein content or in EH and GST enzyme activities were found between corals from the three sites (Table 3.1). However, the activities of BaPH and UDPGT were significantly different in corals from across the environmental gradient from KR (inshore) to SC (offshore) (Table 3.1). The activity of UDPGT at KR exceeded those found in all other samples including the samples for the lunar series in BNP.

The time course of cytochrome P-450, microsomal protein and soluble protein content over the lunar cycle are shown in Figure 3.2. The time course of BaPH, EH, UDPGT and GST over the lunar cycle are shown in Figure 3.3. Large variation between individuals obscured any discernible patterns which may be present. Small sample size prevented the application of statistical analysis.

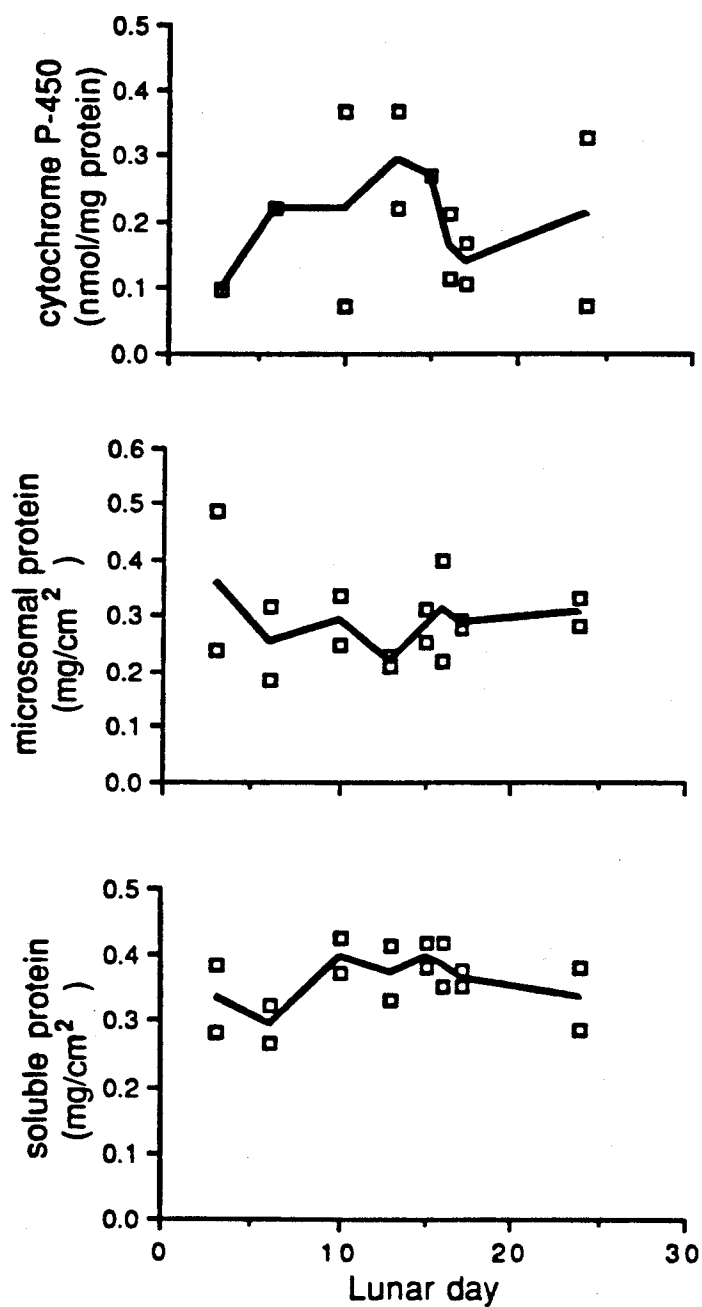
role in spawning (or planulation) events (Pass et al., 1989).

Difficulties Experienced in the 1989 Lunar Sampling

Extraction techniques

Several compounds appeared only at specific times of the cycle in the 1988 sampling. The collections were repeated at shorter time intervals over several months in 1989 to confirm a cyclic nature in these compounds. The change in extraction methods from $\text{CHCl}_3:\text{MeOH}:\text{H}_2\text{O}$ (Bligh & Dyer, 1959) to diethylether was made to enhance the extraction of the $\text{C}_{19}\text{-C}_{21}$ steroid, assumed to be important as biochemical regulators of the cycle. This method was not as quantitative for all lipids or sterols. Using cholesterol as an example, we can see that its relative contribution to the total sterol peak area is highly variable and represents only about 20% of the total peak area. This is in contrast to the 1988 sampling in which cholesterol contributed approximately 40% within a much narrower range of values. Extraction with $\text{CHCl}_3:\text{MeOH}:\text{H}_2\text{O}$ is quantitative for all lipid classes. Because the preparative separation chromatography was adequate to isolate the compounds of interest, the Bligh and Dyer (1959) method would have been the better choice for the latter sampling and would have provided more information correlating sterol profiles to gametogenic biochemistry.

Figure 3.2 Lunar time course of cytochrome P-450, microsomal protein and soluble protein in *Favia fragum*. Collections started on October 11, 1989 (lunar day 10) and continued through mid-November (lunar day 17). The solid line represents the mean value for two corals and squares represent individual data points.

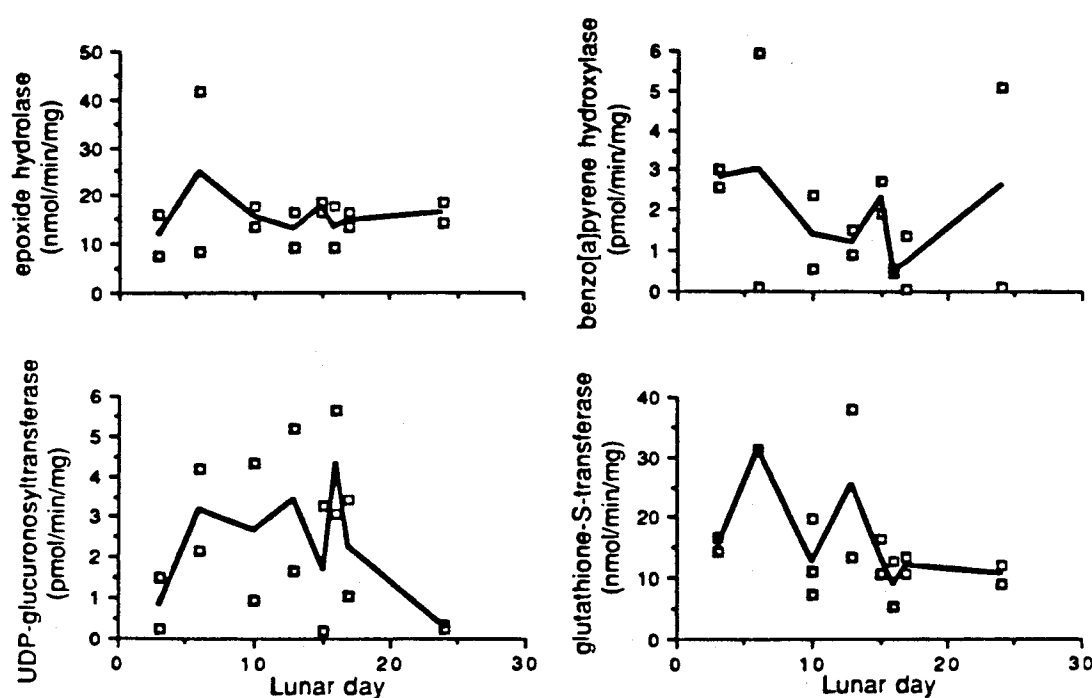


including two scleractinian corals. They found cholest-5-22(E)dien-3 β -ol (Retention time (RT) 46.04) in 11 species including one hard coral (and in the zoanthid, *Palythoa* sp, Kelecom & Solé-Cava, 1982), dihydrocholesterol (RT=47.88) in 5 cnidarians including one hard coral (also another gorgonian, Maquestiau et al., 1978) and desmosterol (RT=48.39) in 2 species. These same three compounds are also found in several species of gorgonian (Popov et al., 1979;1983). Because co-injection of samples and standards was not performed, identifications in *Favia fragum* must be considered very tentative.

1989 Lunar Sampling

Technical problems prevented the generation of an interpretable data set or comparisons between dates. The origin of these problems and some proposed solutions are discussed below. However, one interesting result from these data was the discovery of two peaks which occurred in the chromatogram of the free planula sterols which were absent in the coral animal tissue extract chromatograms (Figure 5.5). Coral colonies collected from days 5-15 of the lunar cycle should have contained numerous planulae. Compounds specific to eggs have been found in soft corals (Bowden et al., 1985; Coll et al., 1989). Extracts of the eggs of the soft coral, *Xenia elongata*, caused contraction of the coral polyp muscles suggesting that egg (or embryo) specific compounds may play a

Figure 3.3 Lunar time course of the enzyme activities of *Favia fragum*. The Phase I enzymes benzo[a]pyrene hydroxylase and epoxide hydrolase and the Phase II enzymes UDP-glucuronosyltransferase and glutathione-S-transferase are shown. Collections started on October 11, 1989 (lunar day 10) and continued through mid-November. The solid line represents the mean value for two corals and squares represent individual data points.



to April, water temperatures are increasing (see Figure 2.1). A similar change in lipids in cnidarians has been noted with changing temperature (Hill-Manning & Blanquet, 1979; Glynn et al., 1985). (It should be noted that comparison of protein data throughout this chapter does not definitively show a seasonal trend in protein data.) The trend of increasing biomass with time may also be indicative of an increase in feeding or an improvement of nutritional status. Szmant-Froelich and Pilson (1980) showed that lipid to protein ratios increase with increased feeding of laboratory corals. The lipid to protein ratio of the first date sampled (0.248, n=5; 3/11/88) was similar to starved *Astrangia danae* while the ratio for the last date sampled in 1988 (0.309, n=4; 4/6/88) falls in the range of *A. danae* which were fed three times a week.

Forty sterol peaks were separated by gas chromatographic analysis. Many of the peaks were only present in a single colony or on a single date. The punctuated appearance of these peaks suggests a correlation to reproduction, a result we attempted to address with the prolonged 1989 lunar sampling. Compounds unique to specific seasonal (reproductive) periods have been noted in the soft corals *Lobophytum compactum* (Bowden et al., 1985) and *Sinularia* sp. (Coll et al., 1989).

Of the peaks coeluting with standards in the 1988 sampling, several had been previously reported in scleractinian corals. Kanazawa et al. (1977) surveyed 22 species of cnidarians

DISCUSSION

Although the metabolism of environmental pollutants by marine invertebrates in other phyla has been extensively studied, very little information exists on the metabolic fate of xenobiotics in the Cnidaria, specifically in corals (Livingstone, 1991). Khan et al. (1972) demonstrated the *in vivo* epoxidation of aldrin, presumably by mixed-function oxidase (MFO) activity, by the freshwater cnidarian, *Hydra*. No MFO activity was detected in the sea anemone, *Metridium* sp. (Lee, 1981; Payne, 1977); however, Lee (1981) suggested that MFO inhibitors may be released upon homogenization of the animal and conditions for enzyme assays may not have been optimal for this species. The results of this study demonstrate the presence of cytochrome P-450 and several Phase I and Phase II xenobiotic metabolizing enzymes in the tissues of the scleractinian coral, *Favia fragum*.

The levels of cytochrome P-450 are similar to, or lower than, those found in many other marine invertebrates (Buhler & Williams, 1989) and lower than those found in many fish species (Collier et al., 1986; Perdu-Durand & Cravedi, 1989; Kennedy et al., 1990). The CO difference spectrum (Figure 3.1) revealed a secondary peak at 420nm. Omura and Sato (1964a,b) have shown this to be an enzymatically inactive breakdown product of cytochrome P-450. While the use of protease inhibitors, albumin, polyalcohols and other

DISCUSSION

1988 Sampling

The intent of the first sampling of the lunarly reproducing coral, *Favia fragum*, was to look for patterns in the sterol profile which might be correlated to reproductive events. Changes in vitellogenic material rich in lipids should have been traceable by this method. In addition to egg lipids, changes in the sterol content of the embryo as it matured could have been detected. Increases in total lipid, neutral lipids and sterol content in reproductive cnidarians has been shown previously (Hill-Manning & Blanquet, 1979; Jennison, 1979; Stimson, 1987; Imbs et al., 1990). Any peaks, in the mammalian steroid region of the chromatogram, which may have been acting as hormones or biochemical regulators of the gametogenic events were of particular interest.

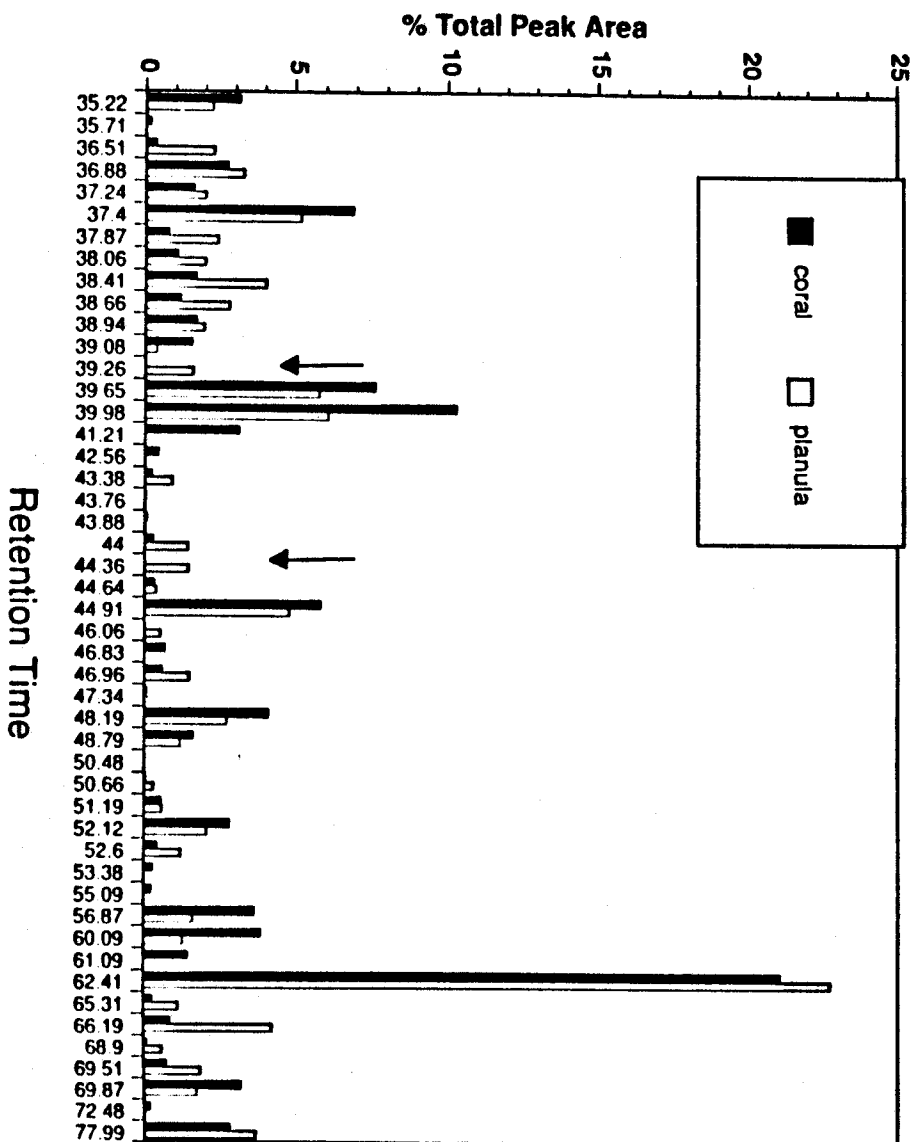
Mean lipid and supernatant protein content increased over time in the 1988 sampling. Total lipid content from March 11, 1988 samples was significantly different from that of the April 6, 1988 (19 ANM) samples. The last date corresponds to several days following planulation and spawning in *Favia fragum* when biomass levels would be expected to be low. In the Pacific brooding coral, *Pocillopora damicornis* (type Y, Richmond & Jokiel, 1984), total lipid content cycles monthly with a peak in total lipids just prior to planulation (Stimson, 1987). Alternatively, the change in biomass seen in this may be reflective of a seasonal trend. From early March

protecting agents can reduce or eliminate P-420 in samples (Lee & Quattrochi, 1987), a P-420 peak may be characteristic of a given species CO difference spectrum (Gilewicz et al., 1984). The conversion of P-450 to P-420 (despite storage in glycerol, this study) may explain lower activities of MFO enzymes *in vitro* (Gilewicz et al., 1984).

The activities of two Phase I xenobiotic enzymes (BaPH and EH) are detectable, with activities similar to, or lower than, other invertebrates (Buhler & Williams, 1989) and fish (Koivusaari, 1983; Perdu-Durand & Cravedi, 1989; Kennedy et al., 1990). It should be noted that in most of these studies, optimized assays for specific tissues which are important in digestion, reproduction or detoxification were examined. The low activities found in *Favia fragum* may reflect that whole animal homogenates were assayed with a standardized technique for determining enzyme activity.

Corals also exhibited Phase II enzyme activity. Levels of UDPGT were similar or lower than reported for fish (Foureman, 1989). However, other studies have shown that many invertebrates conjugate p-nitrophenol to glucose but not to glucuronic acid. The reason for this difference is not clear. GST activity has been reported in other cnidarians (Stenersen et al., 1987) and activities in *Favia fragum* were substantially higher than found in many marine invertebrates (Bend & James, 1978) and some fish species (Perdu-Durand & Cravedi, 1989; Kennedy et al., 1990) but not as high as some

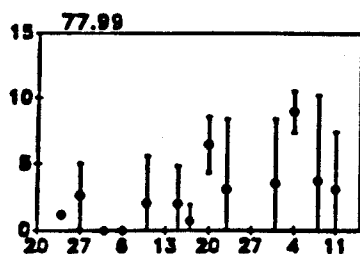
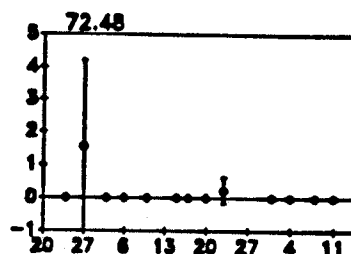
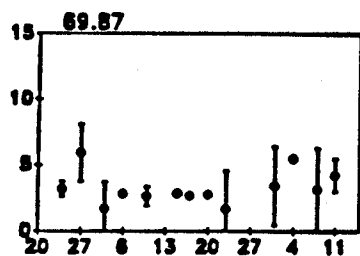
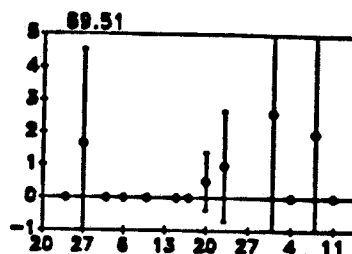
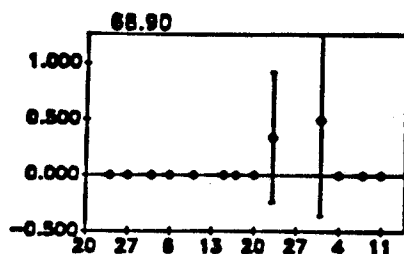
Figure 5.6 1989 *Favia fragum* lunar sampling comparison of percent total peak area between coral values and planula.



species of crabs and mussels (Lee, 1988). Higher GST activities in the corals may be related to protection from high oxygen tensions in coral tissues originating from photosynthesis by zooxanthellae (D'Aoust et al., 1976; Crossland & Barnes, 1977), because one role of glutathione in biological systems is to detoxify reactive oxygen radicals (Ketterer et al., 1983). High levels of another enzyme which scavenges oxygen radicals, superoxide dismutase, are also present in the tissue of marine cnidarians containing endosymbiotic algae as a protectant from oxygen radical damage (Dyken & Shick, 1982; Dyken, 1984).

Many pollutants (e.g. polychlorinated biphenyls and polycyclic aromatic hydrocarbons (PAHs)) are known inducers of cytochrome P-450 and other Phase I and Phase II xenobiotic metabolizing enzymes in mammals and fish. However, the ability of pollutants, such as PAHs, to induce the MFO system in aquatic invertebrates is variable. The induction of invertebrate MFO systems has been shown in several species when exposed to contaminated water or sediments (Lee, 1981; Payne et al., 1983), or to known inducers such as benzo[a]pyrene or Aroclor 1254 (Fries & Lee, 1984). For example, Lee et al. (1979) found that the MFO system in the polychaete *Capitella capitata* was induced upon exposure to crude petroleum. However, Livingstone et al. (1985) reported that while exposure of bivalves to diesel oil resulted in significant increases in digestive gland microsomal P-450

% Total Peak Area

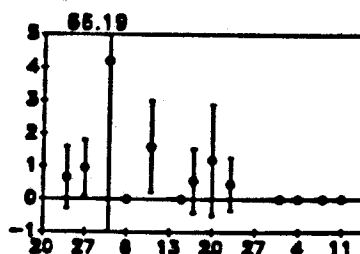
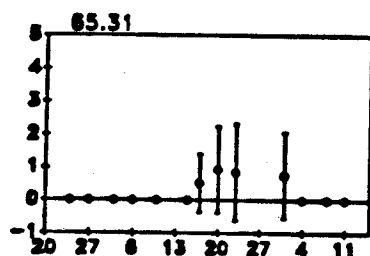
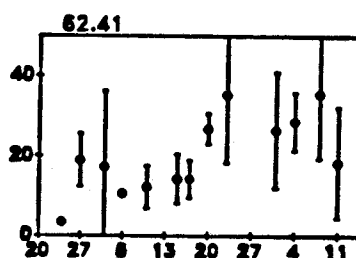
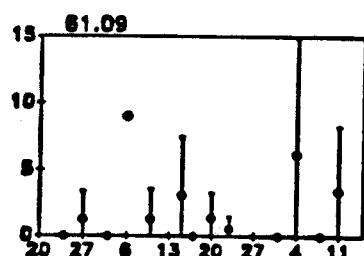
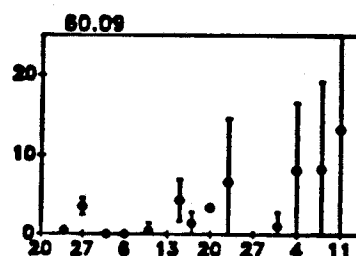
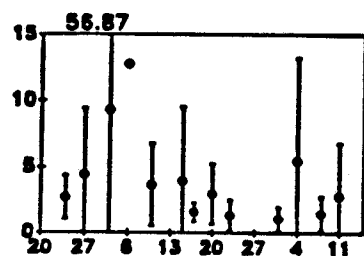
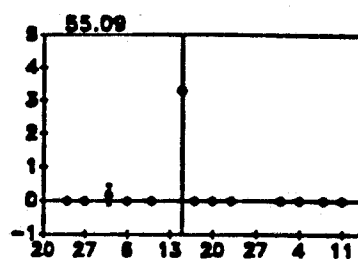
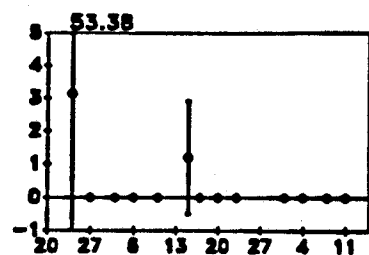


Day After New Moon

levels, these increases were not reflected in BaPH activity. In crustaceans, induction has been shown in some species and not others (James, 1989). Our results showed no significant difference in the levels of cytochrome P-450 or two biotransformation enzymes (EH and GST) between corals sampled from inshore and offshore sites although sediment samples collected concurrently had levels of pesticides (e.g. delta-hexachlorocyclohexane) and polyaromatic hydrocarbons (e.g. pyrene and benzo[a]anthracene) 3 - 19X higher at the inshore site (P. Glynn, unpublished data). However, significantly higher activities of BaPH and UDPGT were found in corals from the most inshore reef. Increased activities may be the result of induction due to exposure to xenobiotics or natural compounds found in terrigenous run-off on these nearshore reefs. As other field studies comparing clean and contaminated sites have concluded (e.g. Payne, 1976; Lee et al., 1982; Stegeman et al., 1986; Van Veld et al., 1990), we suggest these results give indirect evidence that BaPH and UDPGT activity is inducible in aquatic invertebrates such as the coral, *Favia fragum*.

In addition to xenobiotic metabolism, the MFO system is important in the metabolism of steroids, prostaglandins and bile acids (Black & Coon, 1987). Cnidarians are known to have a high lipid content (Bergmann et al., 1956; Patton et al., 1977) which include complex mixtures of sterols (Kanazawa et al., 1977) as well as prostaglandins (Weinheimer & Spraggins,

% Total Peak Area

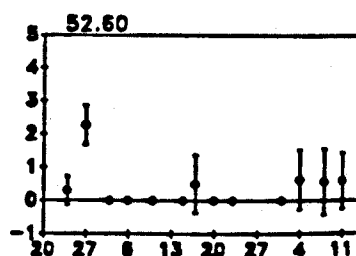
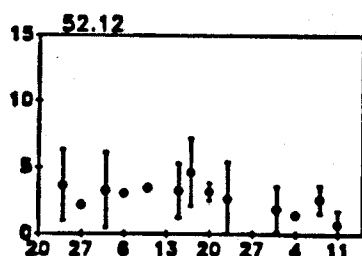
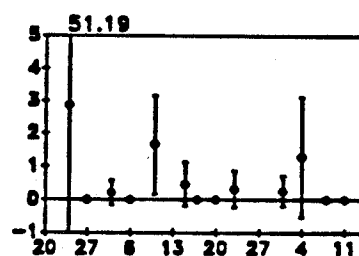
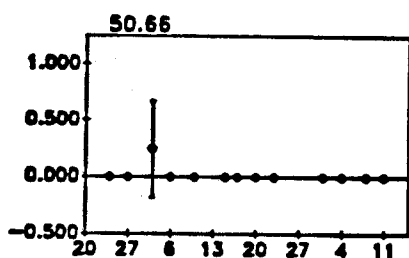
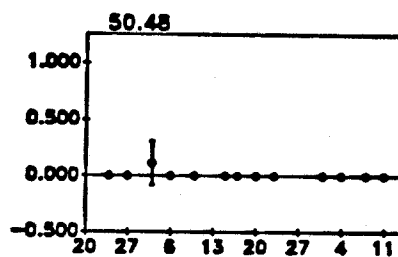
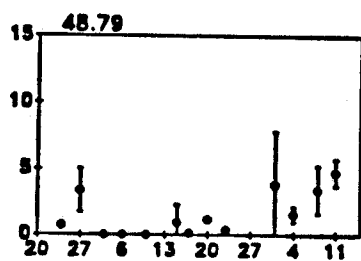
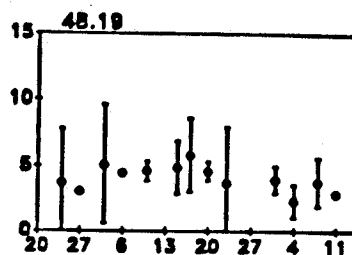
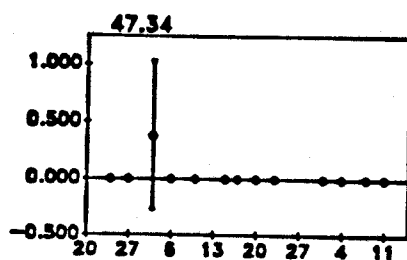


Day After New Moon

1969). Xenobiotics with polyaromatic sterol-like backbones may compete as substrates for MFO enzymes. In theory, a competitive interaction of this type may interfere with lipid metabolism resulting in decreased lipid production and stores or egg volumes, effects seen in corals exposed to petroleum products (Rinkevich & Loya, 1979; Peters et al., 1981; Cook & Knap, 1983; Burns & Knap, 1989).

Conversion of steroid precursors to active hormones in mammals often occurs through cytochrome P-450 mediated metabolism (Norman & Litwack, 1987). Invertebrates also utilize cytochrome P-450 in the β -hydroxylation of both progesterone and testosterone (spiny lobster, James & Shiverick, 1984) as well as in the activation of the steroid β -ecdysone, the hormone responsible for molting (insects, Bollenbacher et al., 1977). Seasonal changes in cytochrome P-450 content have been shown to occur in crustaceans (Lee et al., 1982) and in fish (Koivusaari, 1983; Andersson & Koivusaari, 1985), and have been linked to reproductive changes in these species. The scleractinian coral, *Favia fragum*, has a lunar reproductive cycle which may be dependent upon cytochrome P-450 or associated metabolic enzymes for the regulation of reproduction. The results of this study, however, indicate that while present in measurable quantities, the levels of cytochrome P-450 and enzymes examined have not yet shown significant changes during the reproductive cycle, possibly due to high interindividual variation, small sample

% Total Peak Area



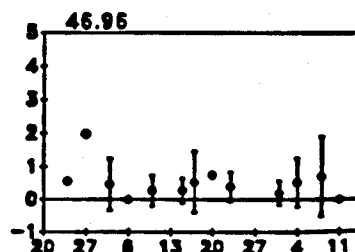
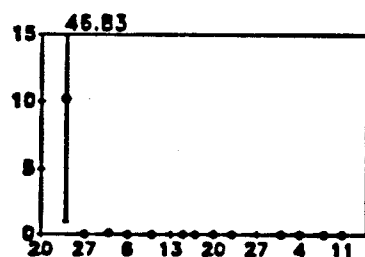
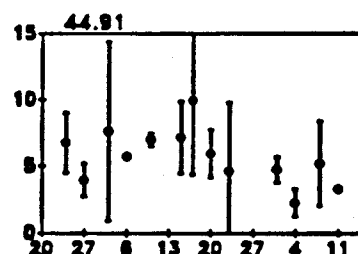
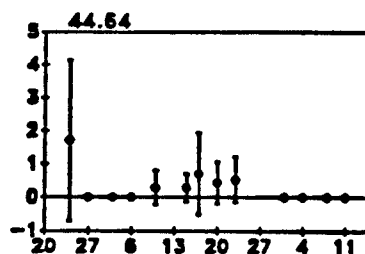
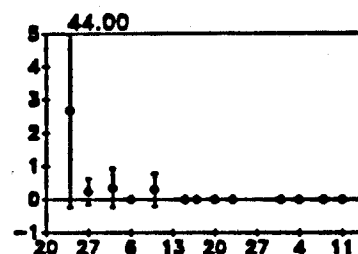
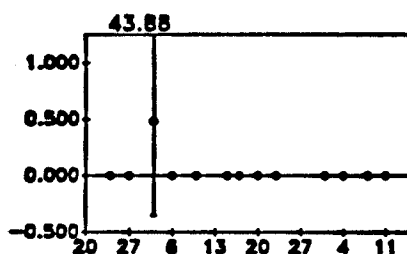
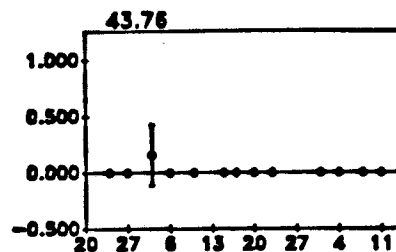
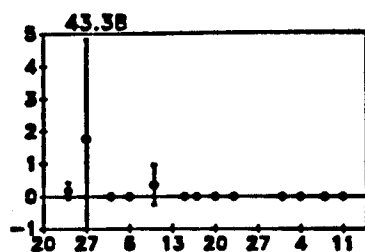
Day After New Moon

size, and possible prior xenobiotic induction, which may have masked any underlying endogenous cycles.

The low levels of activity seen in *Favia fragum* may not be extrapolated to other reef corals. *F. fragum* forms relatively short lived colonies which may allocate the majority of their energy to reproduction rather than growth and maintenance. The reef accreting species are long lived, have greater recuperative powers, and have been shown to detoxify xenobiotics more efficiently than *F. fragum* (Solbakken et al., 1984).

In conclusion, we have demonstrated the presence of cytochrome P-450 and several xenobiotic metabolizing enzymes at very low levels in the coral, *Favia fragum*. Low levels of xenobiotic transforming ability could partly explain the long half-life of many environmental pollutants in corals (Burns & Knap, 1989; Jackson et al., 1989). In view of these findings, and the ecological importance of corals, more research is needed to further characterize and evaluate the role of MFO systems in coral biochemistry.

% Total Peak Area



Day After New Moon

CHAPTER 4

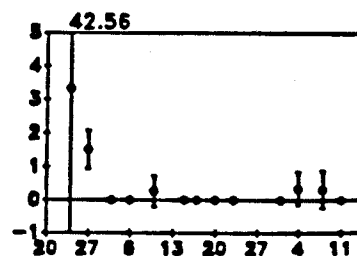
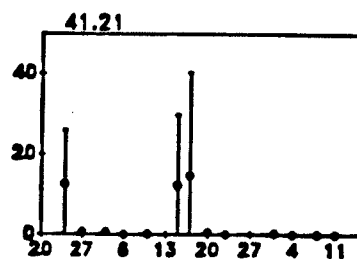
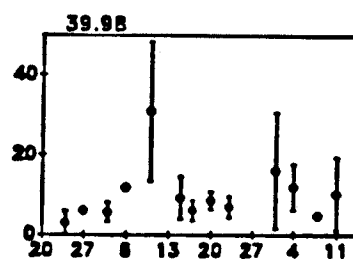
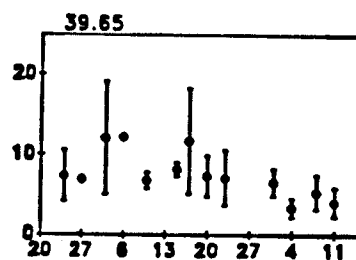
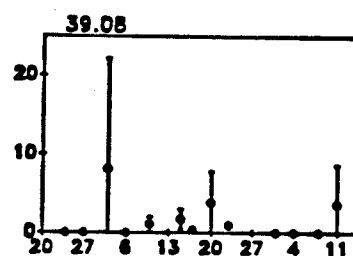
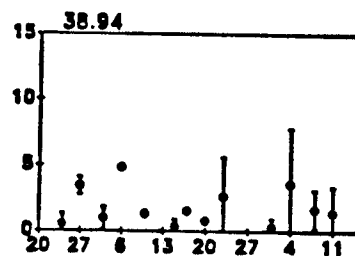
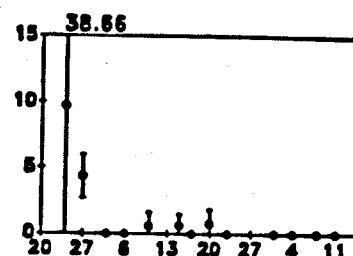
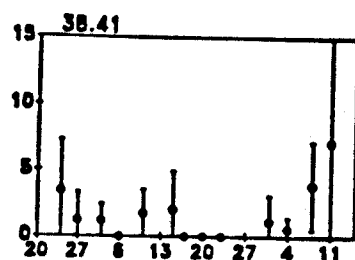
METABOLISM OF PROGESTERONE BY SCLERACTINIAN CORALS

INTRODUCTION

The importance of lipids in cnidarian biochemistry has been recognized for many years (Bergmann, 1962) and is illustrated by their substantial contribution (25-40%) to total dry weight of the organism (Bergmann, 1956; Patton et al., 1977). However, investigations into the sterol component of those lipids has proceeded slowly. The earliest work concentrated on natural products chemistry of these organism (reviewed in Bergmann, 1962) and few sterols were reported. Today, the cnidarians are recognized for the complexity and diversity of the novel sterol compounds they contain (e.g. Kanazawa et al., 1977). Compounds related to vertebrate-type steroids (shorter carbon chain derivatives of sterols) (Higgs & Faulkner, 1977) as well as vertebrate prostaglandins have been found in cnidarians (Weinheimer & Spraggins, 1969).

Early investigations into cnidarian metabolic capabilities often reported an inability to synthesize sterols from known precursors to cholesterol (van Aarem et al., 1964; Ferezou et al., 1972; Walton & Pennock, 1972) or very slow rates of sterol synthesis (Kanazawa et al., 1974; Voogt et al., 1974).

% Total Peak Area

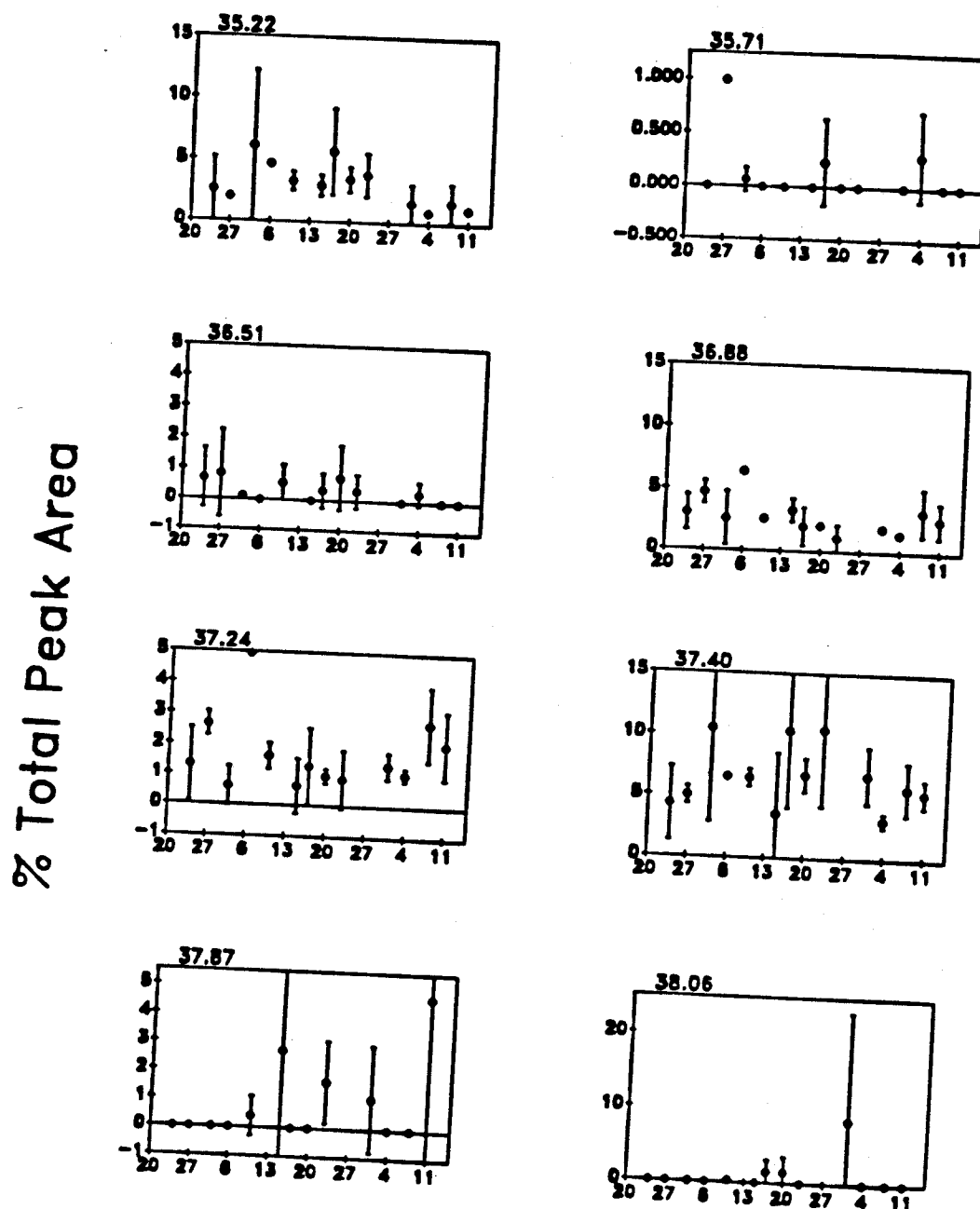


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Within the last decade, interest in the metabolism of lipids and sterols in cnidarians has increased, focussing on the processing of metabolites within the zooxanthellae/animal host symbiosis. Within the intact symbiotic unit, approximately 10% of the total radioactivity incorporated into lipid from radiolabelled acetate and bicarbonate is found in sterol compounds (Crossland et al., 1980; Patton et al., 1983). The ability of the coral symbiotic unit to synthesize sterols from small molecular weight precursors has been attributed to the zooxanthellae, which can produce sterols in isolation from their animal hosts (Patton & Burris, 1983; Kellogg & Patton, 1983). Sterol metabolism in the cnidarian animal tissue in isolation from their zooxanthellae has not been previously reported.

We were interested in the role of sterols in the regulation of reproduction in reef corals. Other higher marine invertebrates (arthropods) have been shown to regulate gametogenic events with vertebrate-type steroids (Voogt et al., 1991) and have demonstrated the ability to synthesize these steroids using biochemical pathways similar to those found in vertebrate groups (Sandor & Mehdi, 1980). How early in the animal kingdom these pathways evolved for control of reproductive events remains an open question due to the lack of information on the metabolism of sterols in cnidarians. For these reasons, the metabolism of progesterone, a precursor to the mammalian androgens and estrogens, was studied in animal

Figure 5.5 1989 *Favia fragum* lunar sampling chromatographic data expressed as percent total peak area. Retention times are listed above each graph. Mean and standard deviation are given.



tissue homogenates isolated from zooxanthellae of several reef corals as well as in the intact symbiotic unit of the coral *Favia fragum*. The results of *in vivo* and *in vitro* incubations demonstrate the existence of a complex pattern of metabolism of vertebrate-type steroids in reef corals.

MATERIALS AND METHODS

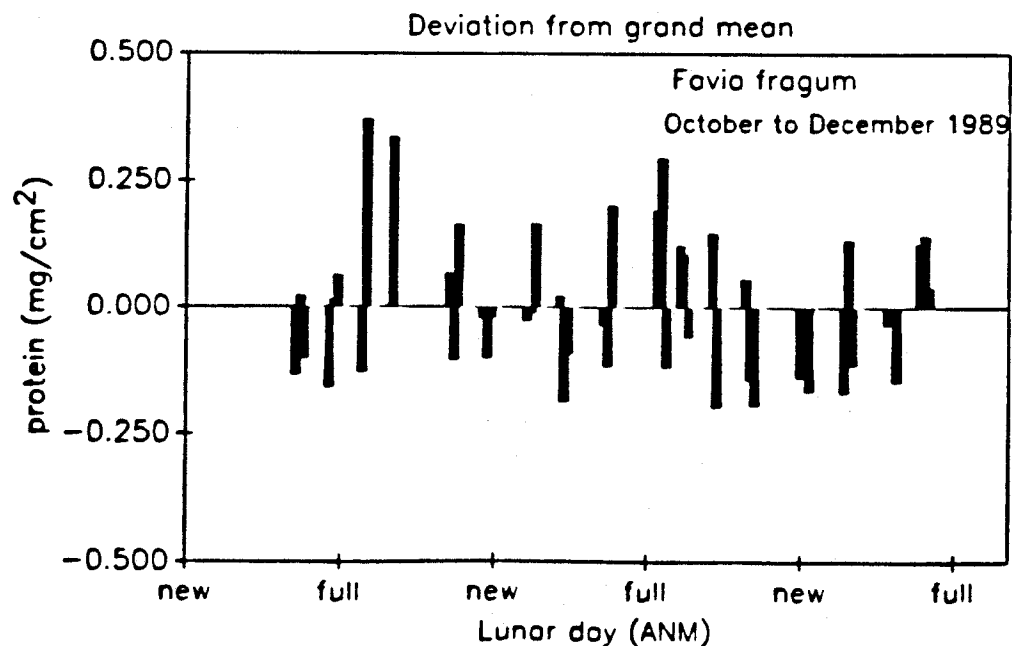
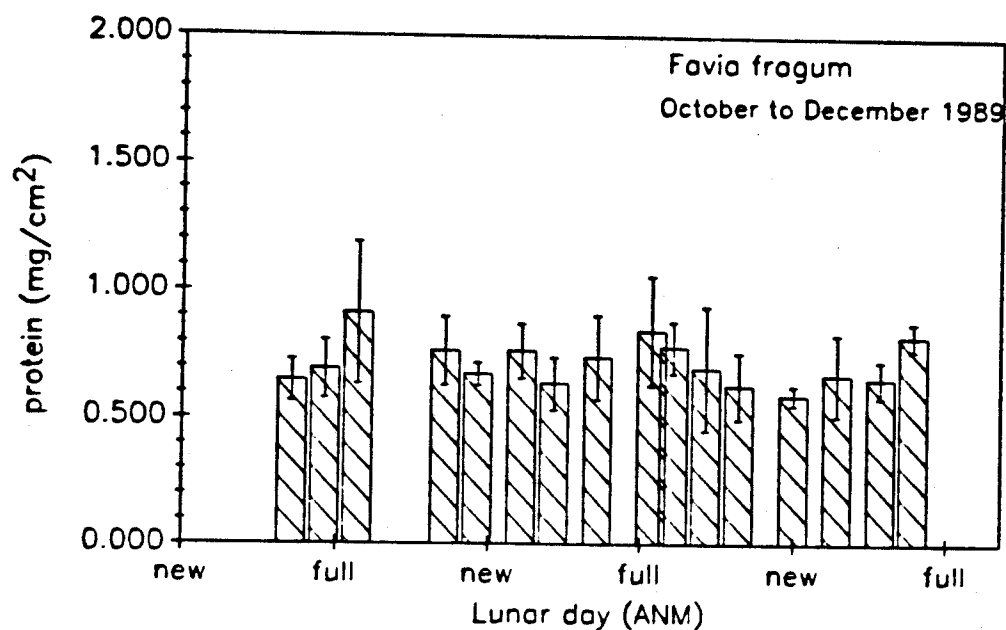
Experimental corals

The small hermaphroditic coral, *Favia fragum*, is particularly suited for a study into the possible cycling of reproductive biochemicals such as steroids because of its lunar cycle of gametogenesis. Reef corals which included the lunar cycling brooder, *F. fragum*, as well as seasonally reproducing species which were either hermaphroditic broadcasters (*Montastrea annularis* and *Diploria strigosa*) or gonochoric broadcasters (*Siderastrea siderea*) were used in this study. The experiment was conducted at a time when most of these species would be in the earliest stages of gametogenesis (Szmant 1986).

In vitro metabolism of ^{14}C -progesterone

Experiments were performed on board the R/V Calanus in March of 1989 during a research cruise to Joulters Cay, Bahamas. *Favia fragum* colonies, 3-6 cm in diameter, were removed from the substrate with a hammer and chisel at 2-3 m depth near Mama Rhoda Rock, Bahamas (25°24'N, 77°55'W). At

Figure 5.4a 1989 *Favia fragum* lunar sampling supernatant protein per surface area. Slightly higher values in corals collected at the time of full moon were not different when plotted as (4b) the deviation from the grand mean.



Joulters Cay, Bahamas (25°16'N, 78°8'W) pieces of *Diploria strigosa* and *Siderastrea siderea* colonies were collected with a chisel and hammer. Two and a half cm diameter cores of *Montastrea annularis* were removed from large heads with a SCUBA-tank powered pneumatic drill fitted with a hole saw from which the guide had been removed. Corals were kept in running seawater on the deck of the ship in shaded aquaria until use.

Tissues were removed from the coral skeleton with pressurized air and 0.2 μm filtered seawater from an artist's airbrush (65 p.s.i.). Tissues were kept on ice throughout this procedure. The coral tissue was homogenized with an Ultra-Turrex for 30 s, centrifuged for 5 minutes at 6000 rpm (1250xg) and the pellet, containing zooxanthellae, skeletal remnants and larger animal fragments, was discarded. The volume of the supernatant containing the animal fraction was measured. A 4.5 ml aliquot was used for the incubation.

Stock 4- ^{14}C -progesterone in benzene (Dupont NEN, 57.2 mCi/mmol) was dried with N_2 and taken up in EtOH and diluted in filtered seawater (0.35% EtOH in assay). No unlabelled progesterone was added. For *Favia fragum* and *Montastrea annularis*, total radioactivity per assay was 0.24 μCi (0.839 μM) progesterone and final concentration of NADPH was 0.75 mM in 5 ml total incubation media. For *Siderastrea siderea* and *Diploria strigosa*, total radioactivity per assay was 0.07 μCi (0.209 μM) progesterone and 0.7 mM NADPH.

The incubation reaction was stopped after three hours with

Table 5.3 Characteristics of 1989 lunar sampling of *Favia fragum*.

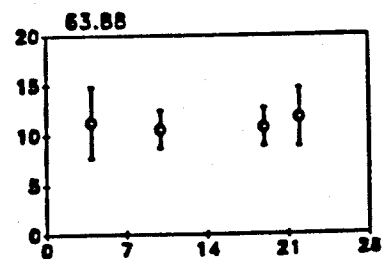
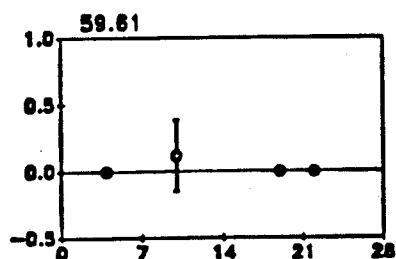
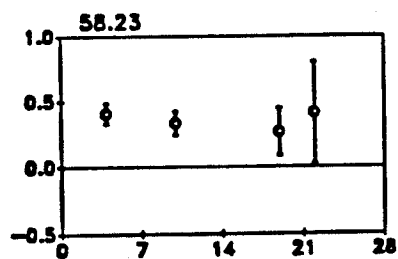
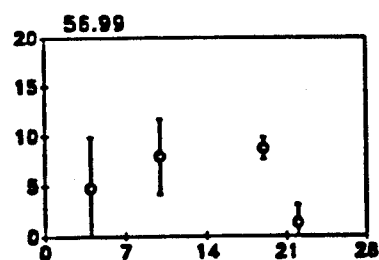
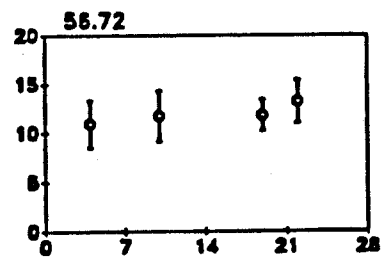
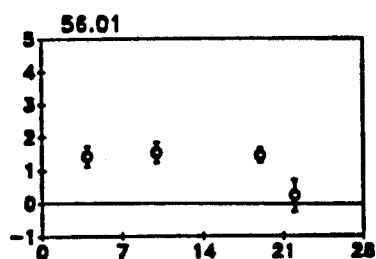
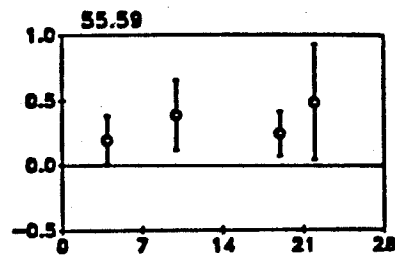
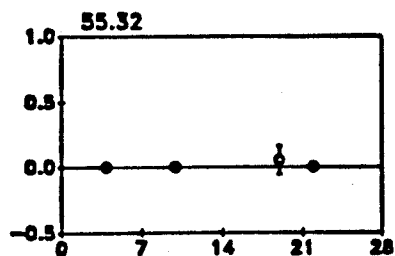
Coral ID	Lunar Day	Surface Area (cm ²)	Supernatant Protein (mg/cm ²)
117	24	8.37	0.615
118	24	9.42	0.883
119	27	11.31	0.697
120	27	19.37	0.619
121	27	9.13	0.697
126	3	4.37	0.692
127	3	24.48	0.707
128	3	12.85	0.886
129	6	6.76	0.742
136	10	12.41	0.685
137	10	16.11	0.765
138	10	16.51	0.725
141	15	6.87	0.911
143	15	11.16	0.601
144	17	10.69	0.844
145	17	10.49	0.828
146	17	5.65	0.661
152	20	13.10	0.867
153	20	9.49	0.523
154	23	12.76	0.774
155	23	16.16	0.577
156	23	12.21	0.526
157	0	11.38	0.581
158	0	10.53	0.630
159	0	10.02	0.554
160	4	7.52	0.552
161	4	10.93	0.853
163	8	7.83	0.685
164	8	9.51	0.704
165	8	7.72	0.573
166	11	9.26	0.845
167	11	13.72	0.498
168	11	8.95	0.249

the addition of CHCl_3 and lipids were extracted in $\text{CHCl}_3:\text{MeOH}:\text{H}_2\text{O}$ (Bligh & Dyer, 1959) for two hours and allowed to phase partition for 6 hours. The aqueous layer was removed and the remaining solvent evaporated under N_2 at 40°C . Dry lipid residues were stored at -18°C until analysis. Lipid residues were dissolved in 500 μl MeOH and filtered through a $0.45\ \mu\text{m}$ membrane filter in preparation for high performance liquid chromatography (HPLC) analysis. Lipid extracts in methanol were injected onto a C-18 (21 cm length) column, using a step gradient of $\text{MeOH}:\text{H}_2\text{O}$ (0-34 min 55:45, 34-59 min 75:25, 59-68 100% MeOH, 68-72 min 75:25, 72-90 min 55:45). While HPLC was able to show the metabolism of progesterone into many compounds, it proved inadequate for resolution of all the metabolites. The use of a methanol, water, and acetonitrile gradient may have provided better resolution of the compounds of interest.

Metabolite analysis

The two dimensional thin layer chromatography (TLC) method of Brown et al. (1988) was used for finer resolution of radiolabelled metabolites. This method can separate 38 known steroid and related compounds. Silica gel pre-coated TLC plates (20 cm X 20 cm., 0.25 mm film thickness, 60 F-254, 5715 Kieselgel EM Scientific) were loaded with approximately 20 μg of 17 steroid standards (Table 4.1, Figure 4.1) and 100 μl of samples in MeOH which had been dried with N_2 at 38°C , and

% Total Peak Area



Day After New Moon

taken up in 50 μ l methylene chloride. Solvent system 1 (methylene chloride:MeOH:H₂O 225:15:1.5 v/v/v) was run once on the plate. For the second dimension, solvent system II (petroleum ether:diethyl ether:acetic acid 48:50:2 v/v/v) was run twice (Figure 4.1).

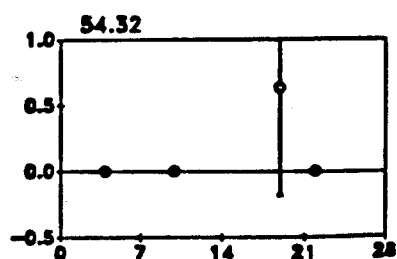
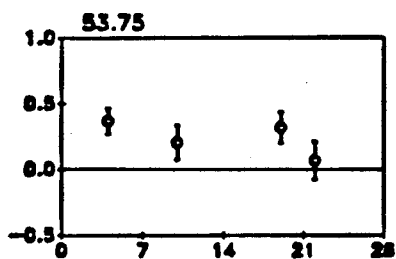
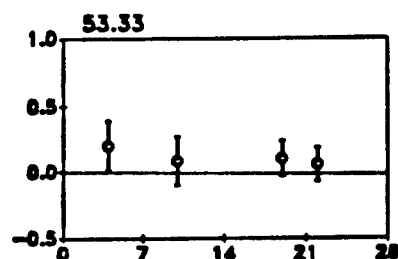
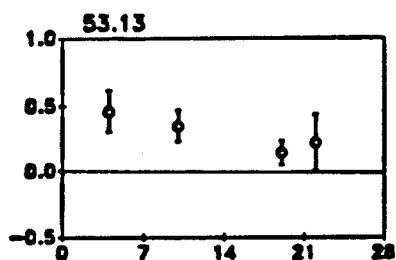
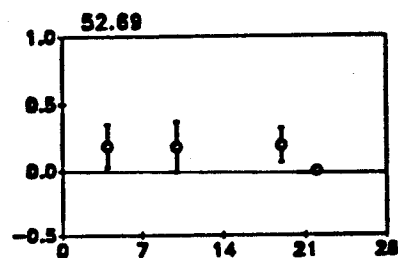
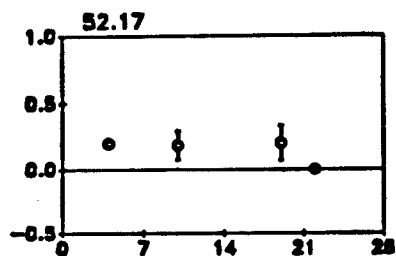
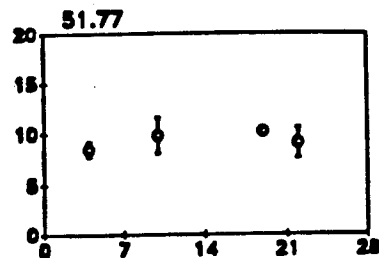
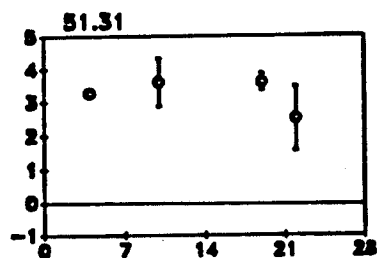
Most steroid standards were visualized by UV (254 nm). The TLC plates were then placed on Kodak OMAT XAR-1 film and exposed for 9 days to detect radioactive metabolites formed by the coral. Origin and progesterone spots were used to orient the film on the TLC plate. Radiolabelled spots were marked directly on the plate with a pencil. Remaining standards were then visualized with a saturated antimony trichloride in acetone spray. TLC plates were heated to 130°C for 5-10 minutes to develop a color reaction.

For each species, a composite map of radiolabelled spots was made. Those spots were removed by scraping the silica off the TLC plate and counting it using 0.8 ml MeOH and 3 ml Ecolume Scintillation cocktail (ICN Biochemical Inc.) on a Beckman LS 1801 scintillation counter.

Calculations and statistics

The percent of total radioactivity per plate per metabolite was calculated. The grand mean value for each metabolite (1-27) was subtracted from each value and the resulting deviation from the mean was divided by the grand standard deviation (Sokal & Rohlf, 1981, p. 110). These data

% Total Peak Area



Day After New Moon

were subjected to a one-way-analysis-of-variance (ANOVA) to compare differences among species. Analysis was performed on a Macintosh computer using Statworks.

In vivo uptake and metabolism of ^{14}C -progesterone

Purification of 4- ^{14}C -progesterone

4- ^{14}C -progesterone (NEN Dupont, 60 mCi/mmol) was purified by two dimensional TLC. An aliquot (30 μl) of 4- ^{14}C -progesterone in benzene was dried with N_2 . 4-Androstenedione and dihydroxytestosterone (DHT) (30 μg each) were added as standards. The steroids were taken up in 100 μl methylene chloride and spotted onto silica gel TLC plates. Two dimensional chromatography was performed according to Brown et al. (1988).

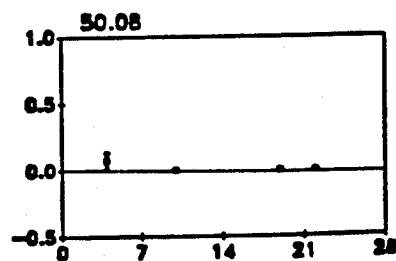
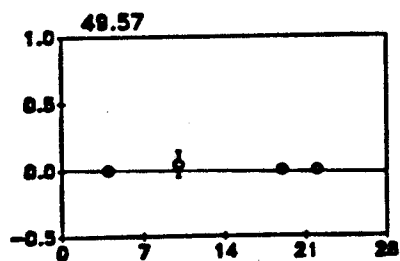
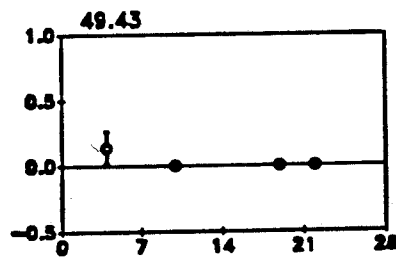
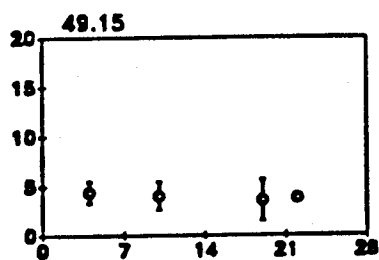
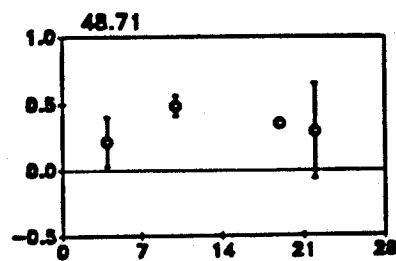
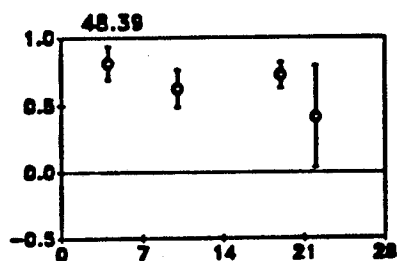
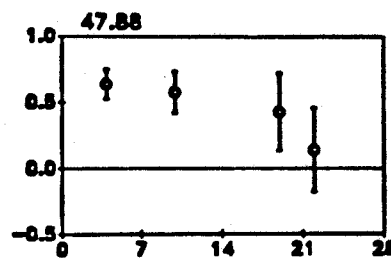
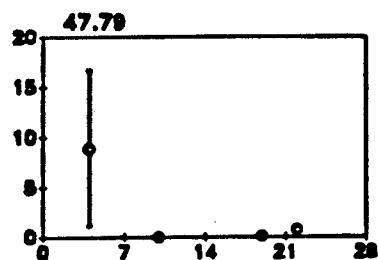
Progesterone and androstenedione were visualized with UV at 254 nm. The progesterone spot was generously scraped. Other spots on the plate were visualized with a light spray of a saturated solution of antimony trichloride in acetone. DHT and a spot corresponding to testosterone were stained.

4- ^{14}C -progesterone was eluted off the silica gel with 3 ml MeOH, followed by two 3 ml washes with CHCl_3 . The solution was dried over N_2 at 35°C.

In vivo incubation

Purified 4- ^{14}C -progesterone was taken up in 300 μl 1% vol/vol Tween 80 (polyoxyethylenesorbitanmonooleate) / H_2O and

% Total Peak Area



Day After New Moon

further diluted in 5 ml 0.45 μm filtered autoclaved sterilized seawater. No unlabelled progesterone was added. A portion was sampled for LSC. One ml of the solution was added to 100 ml sterile seawater in 250 ml beakers (Tween 80 = 0.006% of final solution). One coral colony was placed in each of four beakers. One beaker containing only sterile seawater and progesterone served as a control. Surface aeration and circulation was provided by an aquarium pump connected to a multiple valve outlet to which glass pipets had been attached. The incubation media was sampled (0.5 ml) at various intervals.

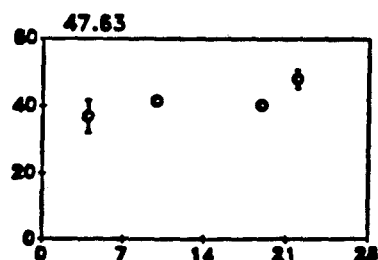
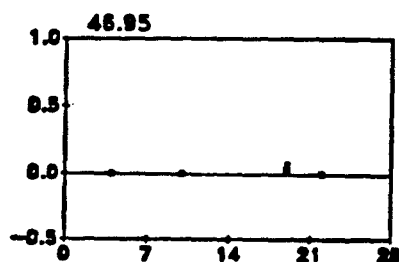
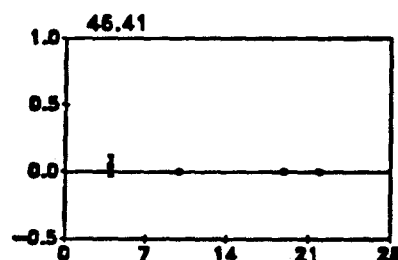
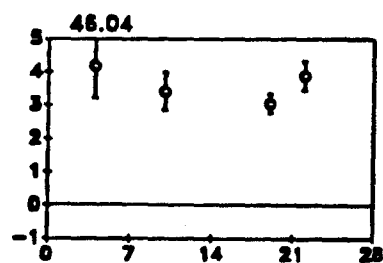
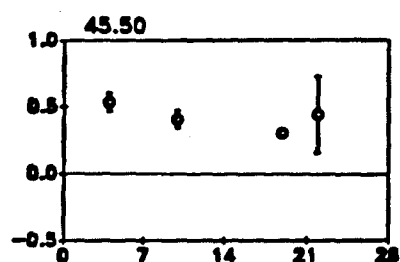
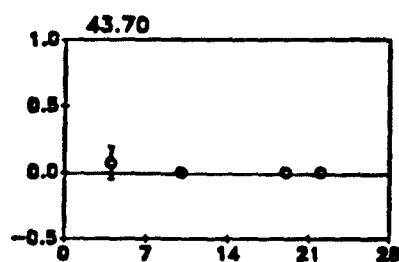
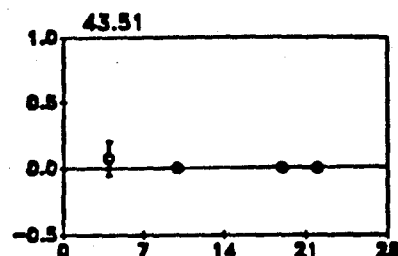
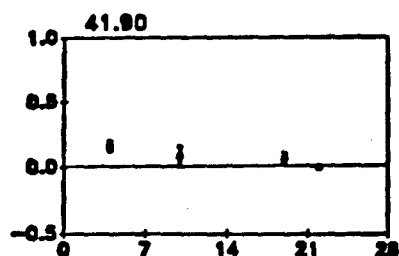
Tissue processing

Corals were removed from the incubation media at 1.5 and 3 hours. Tissues were removed with the airbrush procedure as described earlier. Animal tissues were extracted according to Bligh and Dyer (1959) as were aliquots of the control seawater.

The zooxanthellae pellets, isolated by centrifugation, were washed with filtered seawater and gently mixed in a conical glass tissue grinder to gently separate the algal cells from mucus. The suspensions were then centrifuged at approximately 1500xg for 5 minutes. This was repeated three times to remove animal tissue remnants. Zooxanthellae preparations were extracted as above.

The resulting extracts were plated on two-dimensional TLC

% Total Peak Area



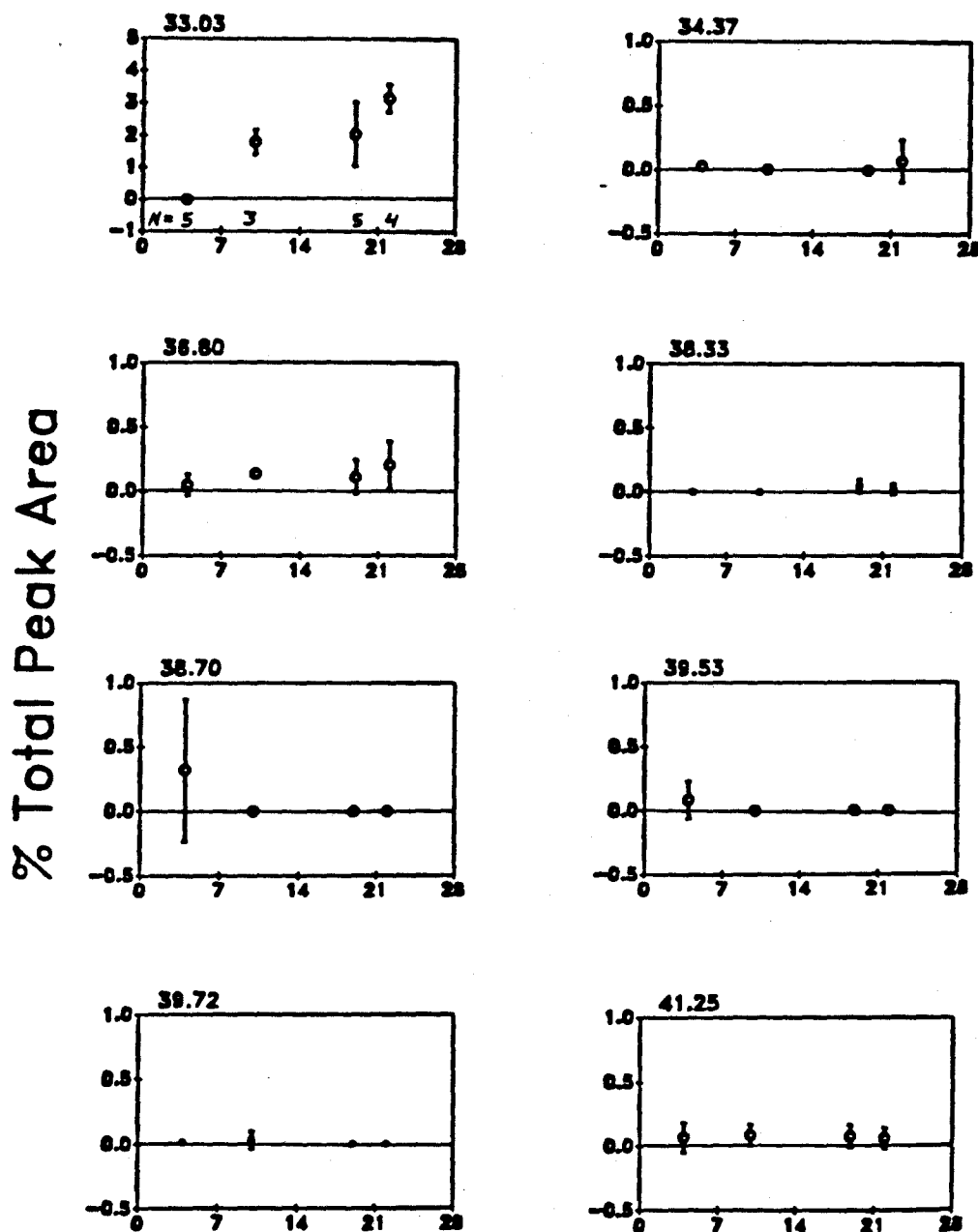
Day After New Moon

Table 4.1 Thin layer chromatography standards. Steroid standards run with samples from the *in vitro* incubation of ^{14}C -progesterone with four scleractinian coral species on silica gel thin layer chromatography plates (RF values from Brown et al., 1988).

ID	RF1	RF2	Std #
Estriol	0.09	0.14	a *
5-Androstenetriol	0.13	0.15	b *
16 α OH-testosterone	0.17	0.06	c *
11-Deoxycortisol	0.41	0.21	d *
5-Androstenediol	0.41	0.61	e
Estradiol	0.42	0.75	f
16 α OH-Androstenedione	0.55	0.23	g
11 β OH-Androstenedione	0.56	0.32	h *
21-OH-Pregnenolone	0.52	0.48	i
Testosterone	0.57	0.51	j *
17 α OH-Progesterone	0.67	0.54	k *
Dehydroisoandrosterone	0.60	0.70	l
Pregnenolone	0.62	0.77	m *
Estrone	0.68	0.84	n *
Cholesterol	0.71	0.90	o *
4-Androstenedione	0.79	0.56	p *
Progesterone	0.82	0.71	q *

* Used as standards for *in vivo* study

Figure 5.3 1988 *Favia fragum* sampling chromatographic data expressed as percent of total peak area. Retention times are listed above each graph. Mean and standard deviation are given with sample size listed in the first graph.



with standards. The standards were visualized with UV at 254 nm. The air dried plates were then subjected to autoradiography on Kodak X-OMAT film for 21 days. Elution position of radioactive spots were compared with standards.

RESULTS

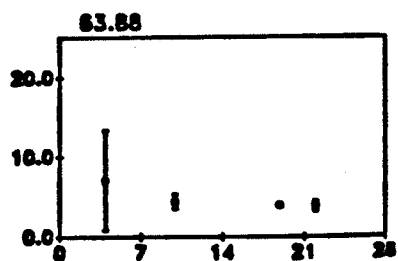
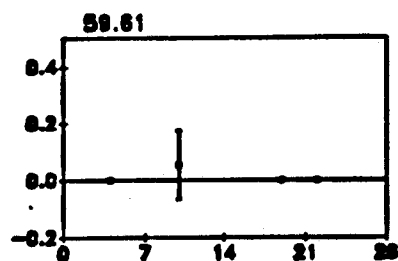
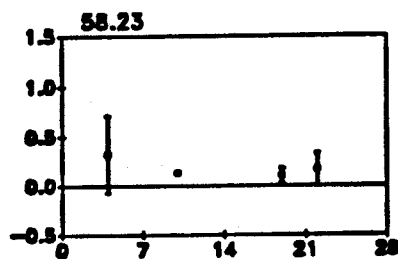
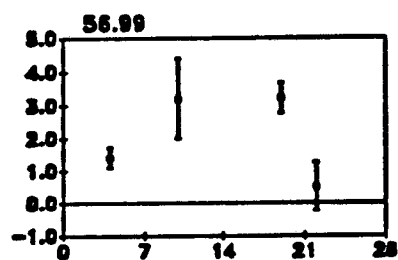
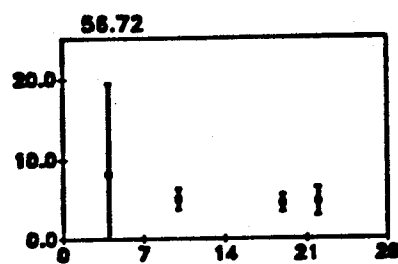
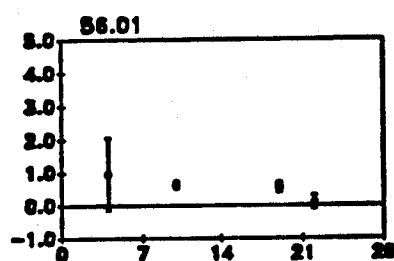
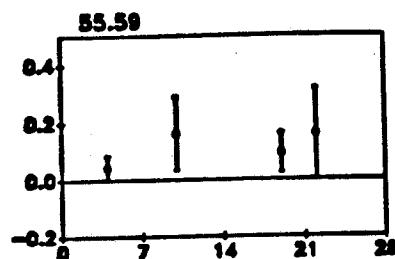
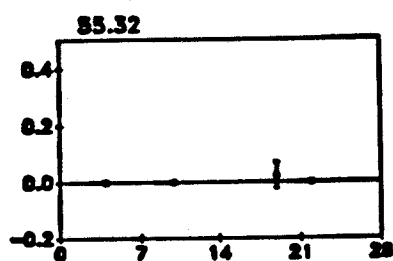
In vitro metabolism of ^{14}C -progesterone

Twenty percent of the total radiolabel was detected in the MeOH insoluble fraction. In the MeOH soluble fraction, less than 25% of the progesterone was metabolized to other products, indicating that the enzymatic pathways were not substrate limited. The *in vitro* conditions used may not have been optimal for the enzymatic pathways to operate efficiently or these pathways operate slowly on these types of substrates.

A total of 27 radioactive metabolites were found in the four species (Figure 4.2). Only 5 metabolites co-migrated with known standards. None of the other radiolabelled metabolites could be visualized with 254 nm UV and therefore they were not 3-keto- Δ^4 -steroids. However the inability to visualize with UV may have been due to inadequate quantities of compound although Brown et al. (1988) claim quantities as small as 5-10 μg are enough to visualize.

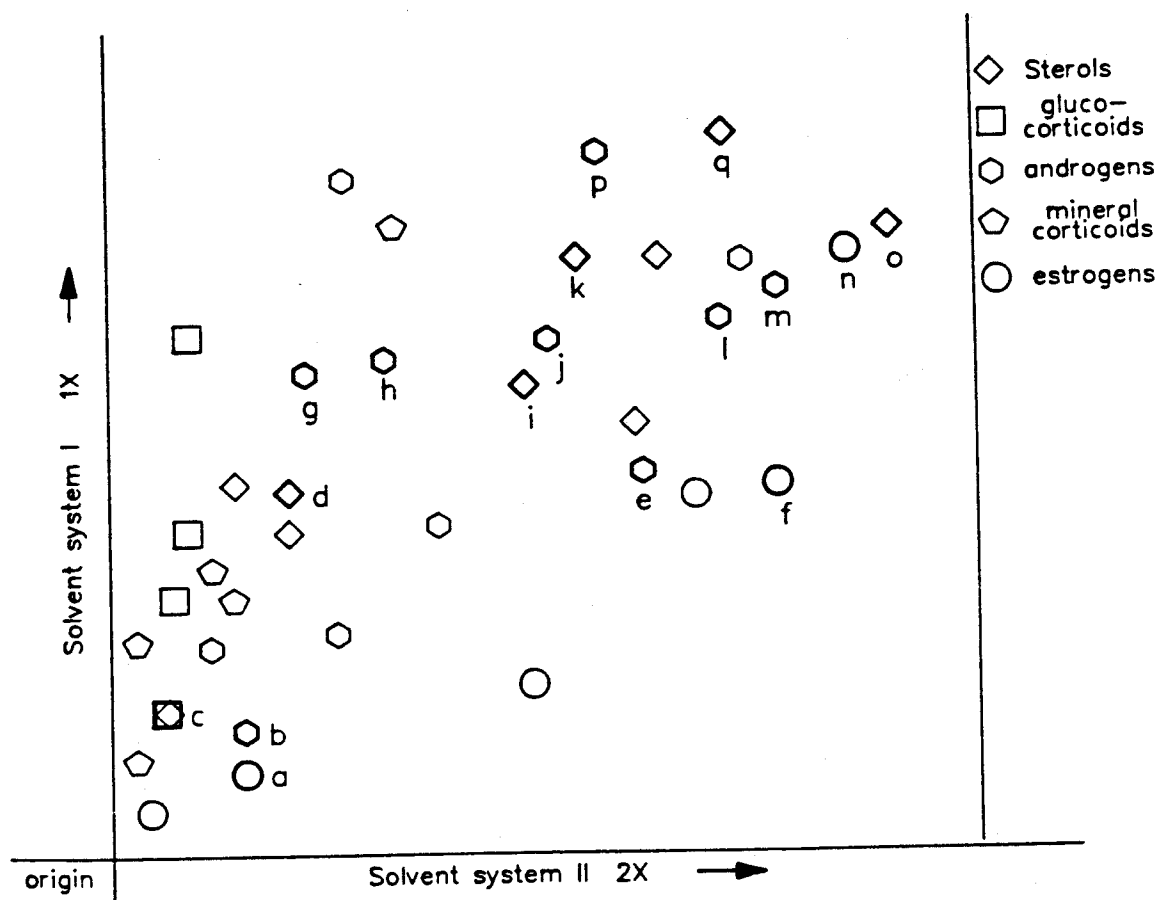
Each species differed in its metabolite profile (Figure 4.3). Metabolites 0-4 and 6-12 were present in all species while 5 and 13 were present in all species except *Siderastrea siderea*. Of the common metabolites, the percent of total

Cholesterol units/mg protein

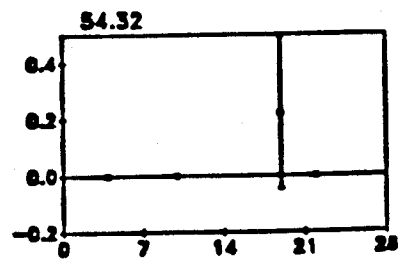
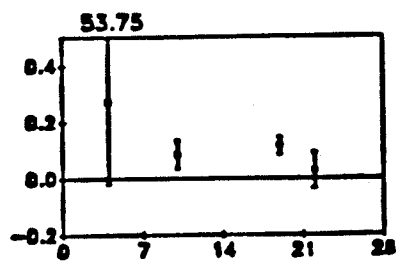
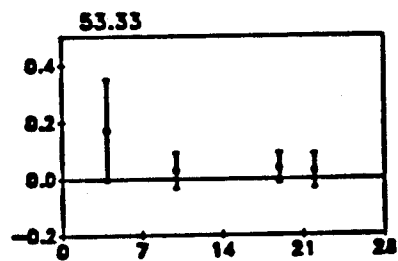
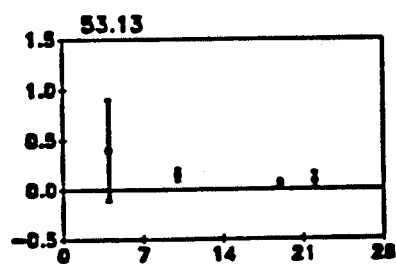
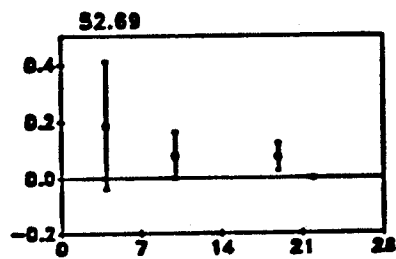
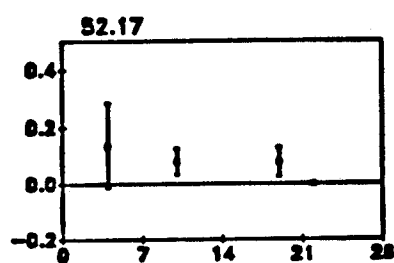
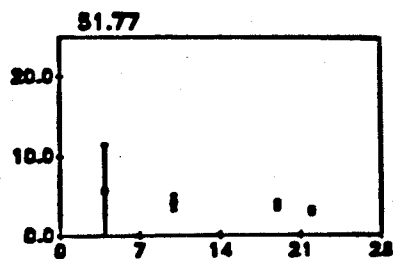
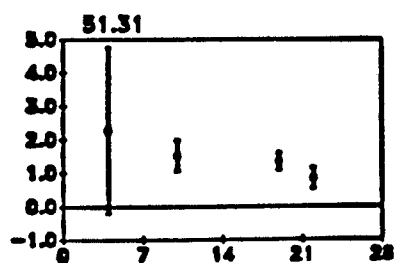


Day After New Moon

Figure 4.1 Diagrammatic representation of two-dimensional TLC of sterol standards (after Brown et al., 1988). Bold and lettered standards are those used in this study.

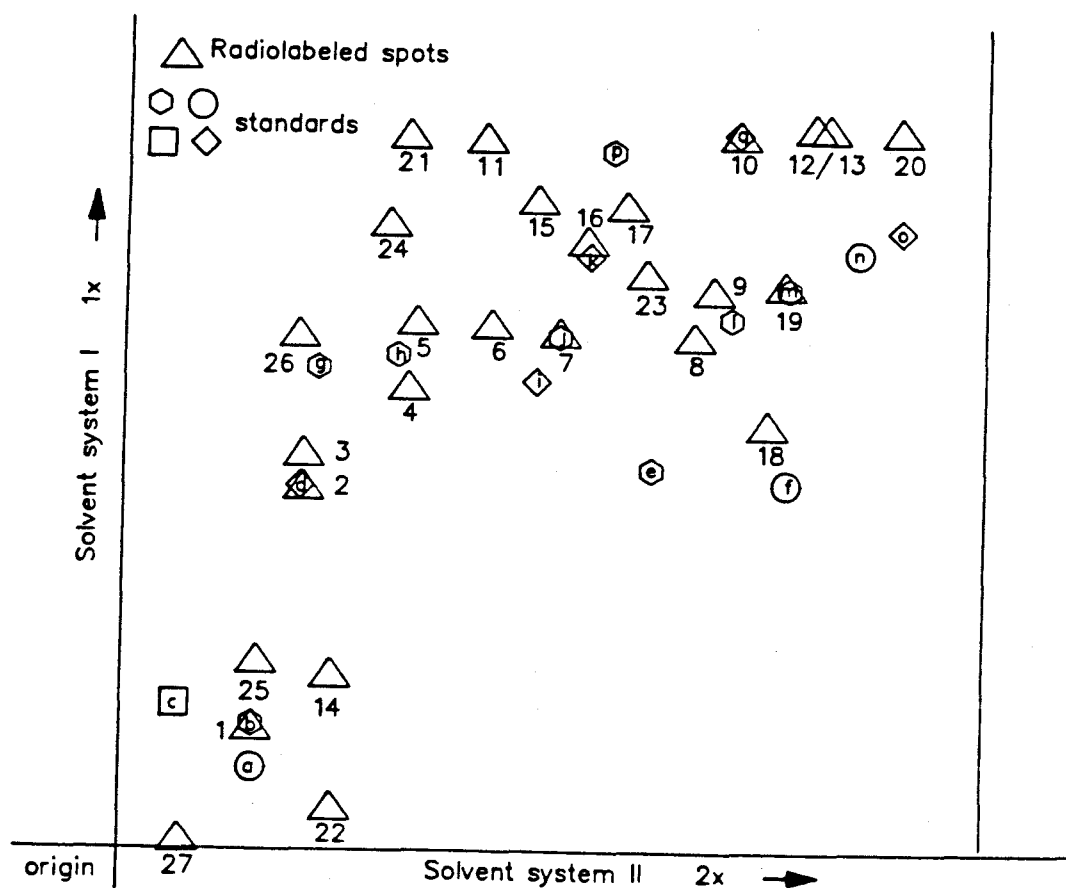


Cholesterol units/mg protein

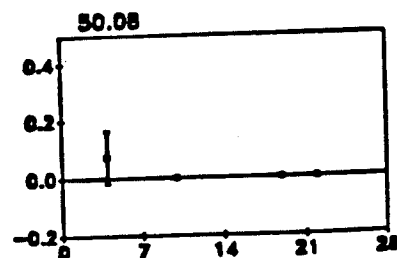
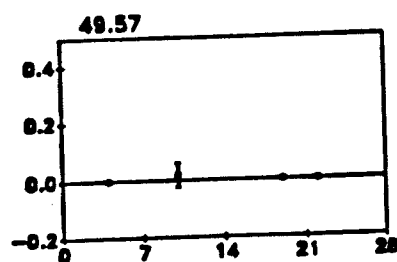
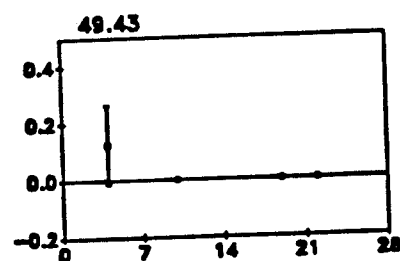
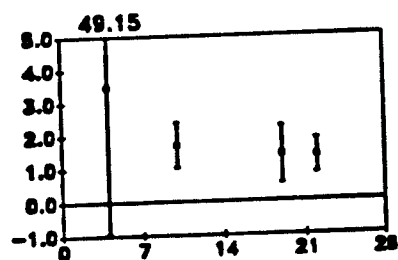
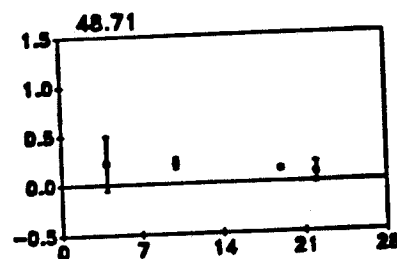
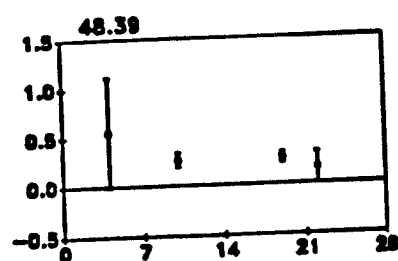
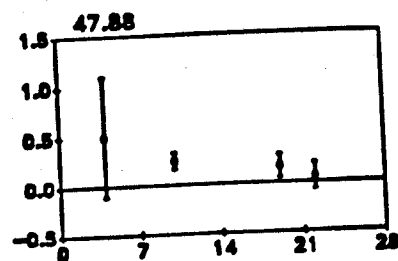
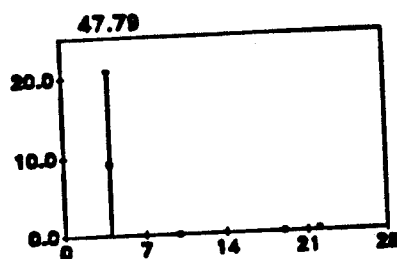


Day After New Moon

Figure 4.2 Diagrammatic representation of two-dimensional TLC of radiolabelled metabolites (triangles Δ) of ^{14}C -progesterone. In vitro incubation with the animal tissues of four species of scleractinian coral (*Favia fragum*, *Montastrea annularis*, *Diploria strigosa*, and *Siderastrea siderea*) produced the metabolites. Standards co-migrated with samples are shown as various polygons (refer to Table 4.1 for standard identification).

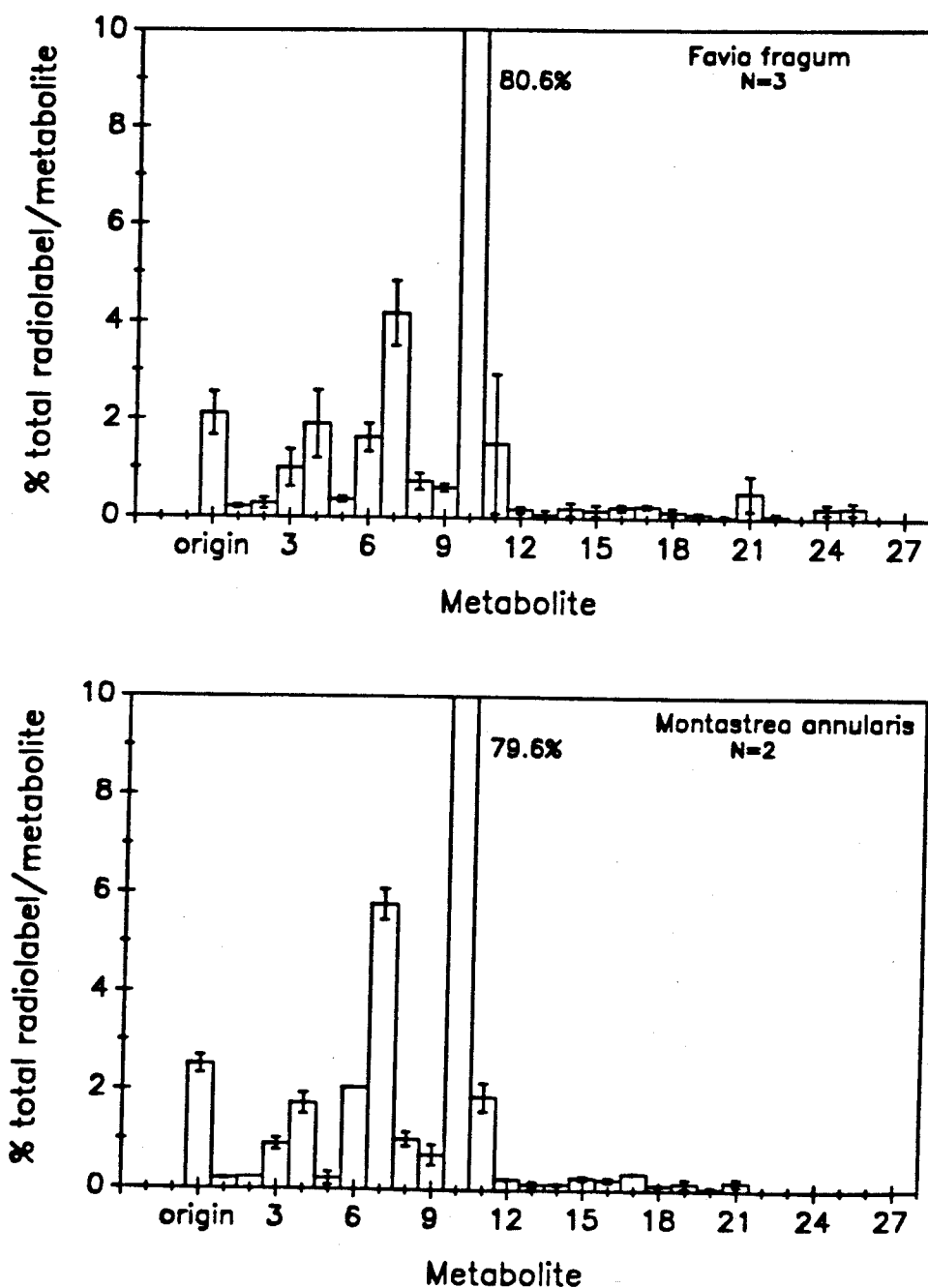


Cholesterol units/mg protein

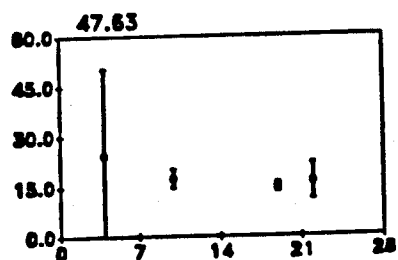
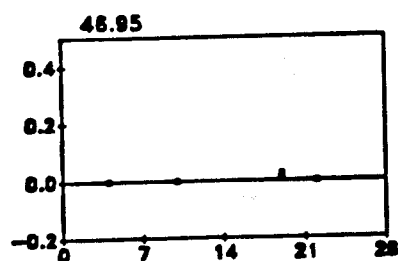
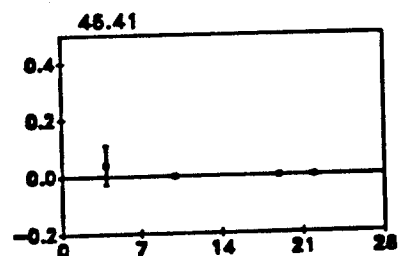
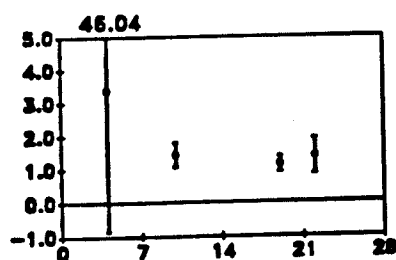
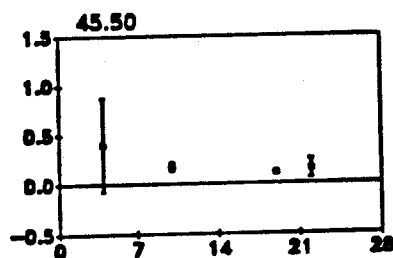
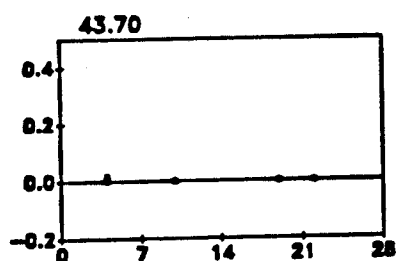
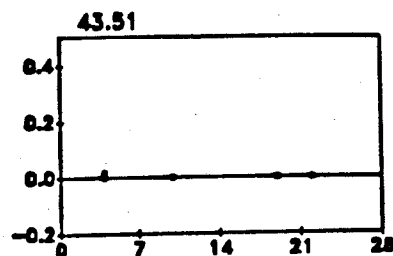
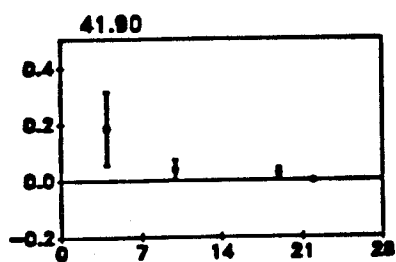


Days After New Moon

Figure 4.3 The distribution of total radioactivity (expressed as a percent) in each metabolite produced by coral tissue homogenates incubated *in vitro* with ^{14}C -progesterone. The incubations were run with four species of scleractinian coral (*Favia fragum*, *Montastrea annularis*, *Diploria strigosa*, and *Siderastrea siderea*). Error bars represent one standard deviation. Species and samples size are given. Metabolite # 1, 2, 7, 10, 16 and 19 comigrate with 5-androstenetriol, 11-deoxycortisol, testosterone, progesterone, $17\alpha\text{OH}$ -progesterone and pregnenolone respectively.



Cholesterol units/mg protein



Days After New Moon

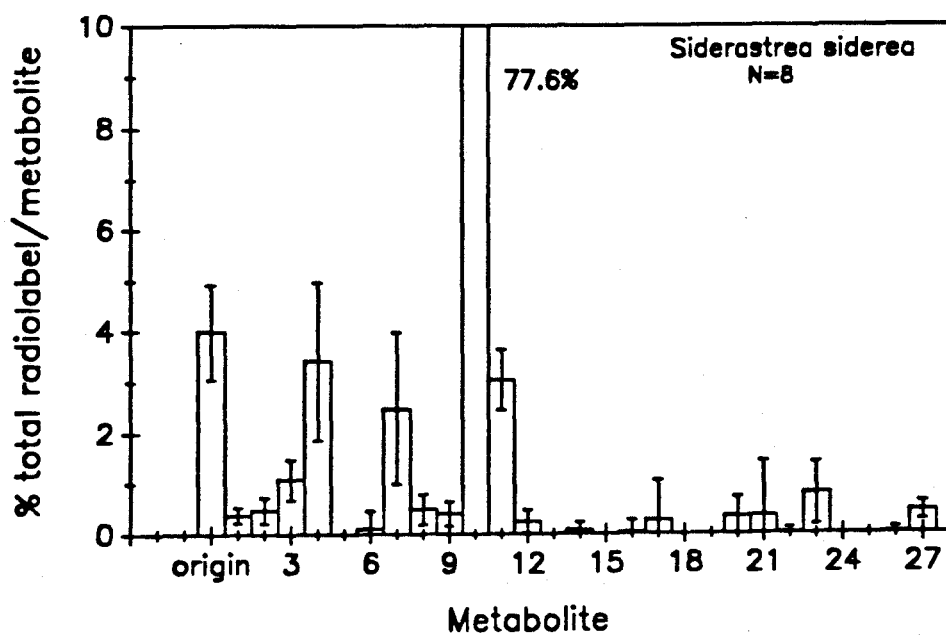
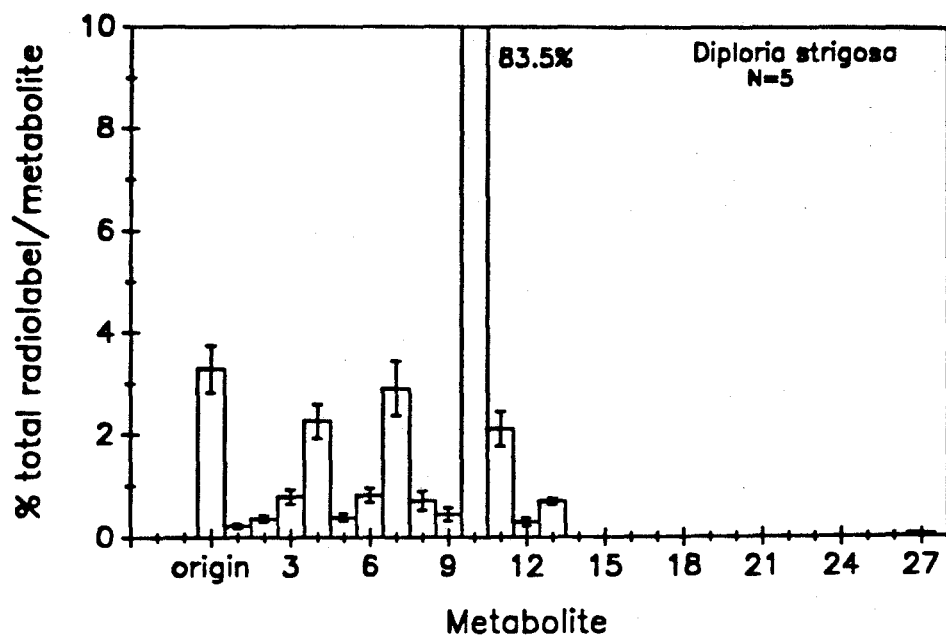


Figure 5.2 1988 *Favia fragum* sampling chromatographic data expressed as cholesterol units per mg supernatant protein. Retention times are listed above each graph. Mean and standard deviation are given with sample size listed in the first graph.

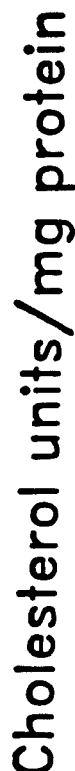
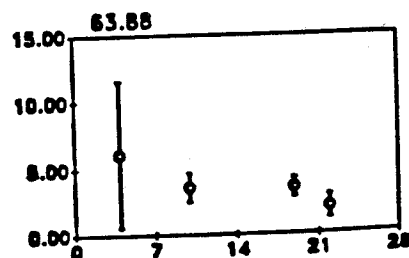
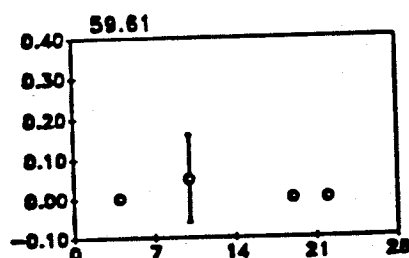
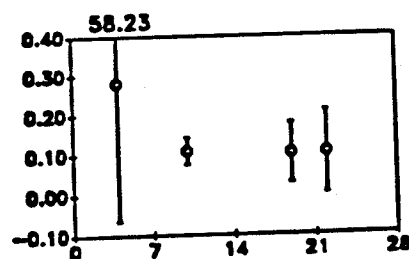
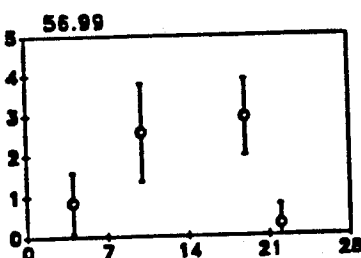
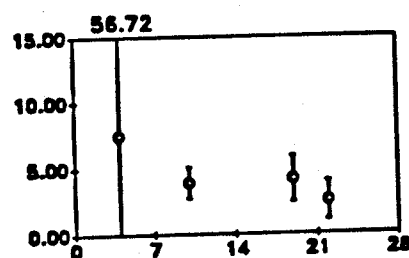
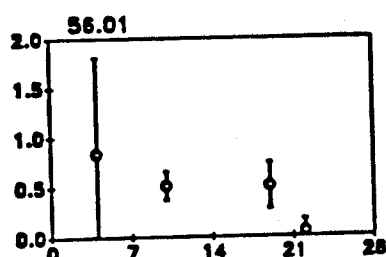
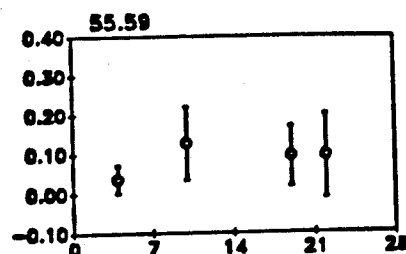
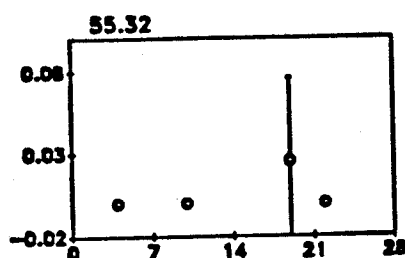


Table 4.2 Metabolites from in vivo incubation of *Favia fragum* with ^{14}C progesterone. The distribution of radiolabelled metabolites among *Favia fragum* colonies incubated with for 1.5 hours (FF_{11} and FF_{12}) and 3 hours (FF_{31} and FF_{32}) and their zooxanthellae is shown. Seawater controls contained only spot #10 = progesterone.

ID	FF_{11}	FF_{12}	FF_{31}	FF_{32}	FF_{11} ZOOX	FF_{12} ZOOX	FF_{31} ZOOX	FF_{32} ZOOX	STD #
1			X						b
2	X	X				X			d
4	X	X							
8	X	X	X	X	X			X	
10	X	X	X	X					q
12	X	X	X			X	X		
13	X	X	X	X	X	X	X	X	
17					X				
18			X			X			
19		X		X		X		X	m
20		X	X	X					
23		X							
24				X		X			
25	X								
26					X	X			
28						X			
29		X				X			
30			X						
31			X						
32	X	X	X			X	X	X	
33	X	X		X	X	X	X	X	
34	X	X		X					
35	X	X	X	X	X	X	X	X	
36	X								
37	X	X	X	X		X	X	X	
38		X				X			e

Cholesterol units/cm²

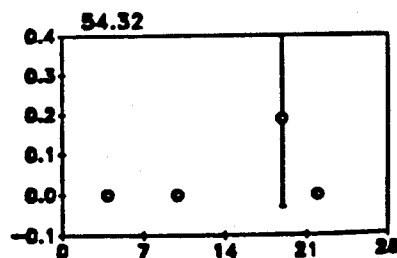
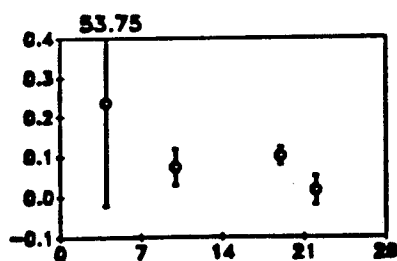
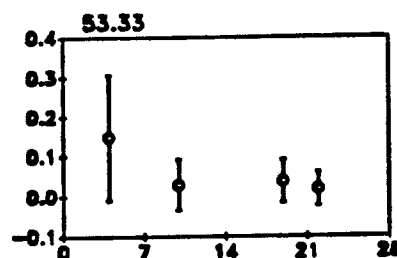
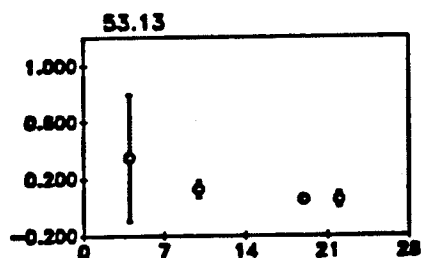
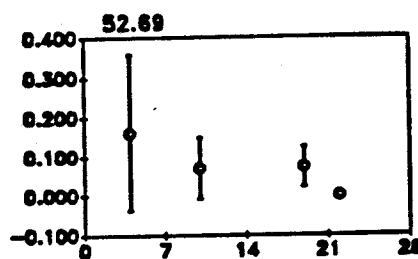
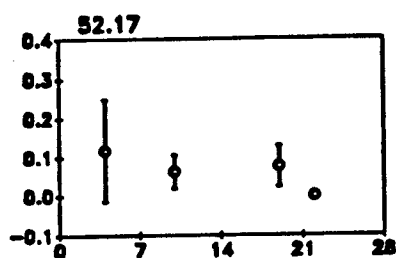
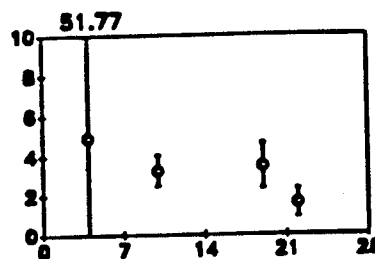
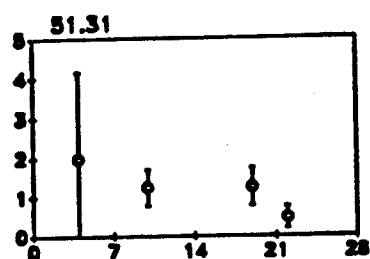
Days After New Moon

radioactivity only differed for the origin (ANOVA, $p < 0.039$) and metabolite 7 ($p < 0.025$). *Diploria strigosa* had the fewest metabolites with 12 (not including the origin and progesterone) while *Favia fragum* had the most metabolites (23). *S. siderea* and *Montastrea annularis* produced 19 and 20 metabolites, respectively.

Of the five metabolites exactly co-migrating with standards (Figure 4.2), testosterone (#7) was the most prevalent metabolite at 3-5% of the total radioactivity scraped from the plate. Metabolites co-migrating with 5-androstenetriol (#1) and 11-deoxycortisol (#2) were present in all species. 17 α OH-progesterone (#16), the metabolic intermediate between progesterone and testosterone, was present in all but *Diploria strigosa*. A radiolabelled spot co-migrating with pregnenolone (#19) was present in the metabolites of *Favia fragum* and *Montastrea annularis*.

In vivo uptake of ^{14}C -progesterone

The initial concentration in the incubation medium was $3766 \pm 215 \mu\text{g}/^{14}\text{C}$ progesterone/l. These concentrations were constant in the control vial: less than 5% of the added ^{14}C progesterone was lost due to adsorption onto the walls of the beakers during the three hours of exposure. TLC of lipid extracts of the seawater controls had no radiolabelled metabolites. An average of 19.5% of the available

Cholesterol units/cm²

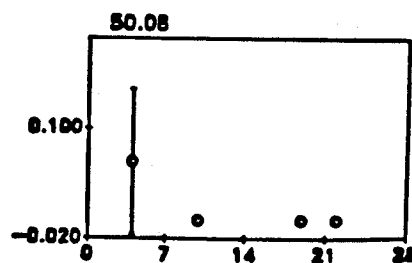
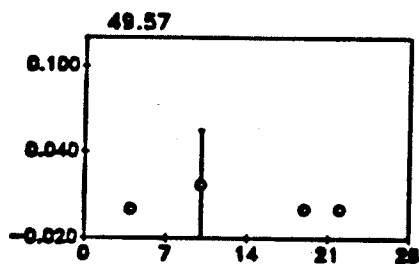
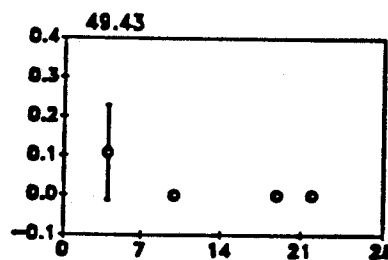
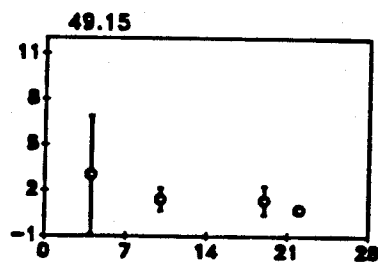
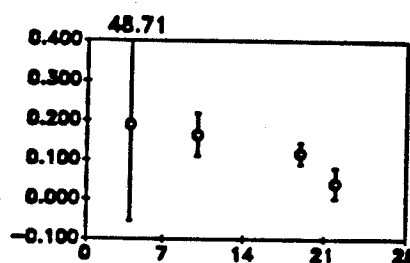
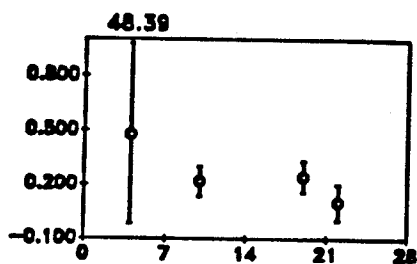
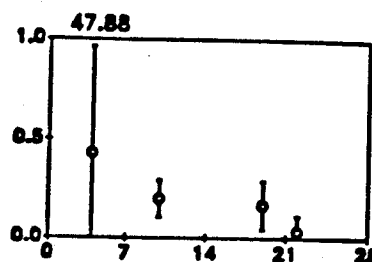
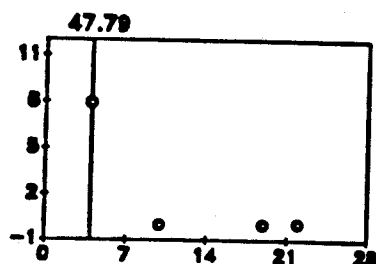
Days After New Moon

radiolabelled was removed from the incubation media. The average uptake rate, estimated from the disappearance of label from the incubation media, was 6.33 ± 7.57 ng progesterone/cm²/hr at a range of concentrations from 2900 to 4000 ng progesterone/l (1.18 ± 1.44 nCi/cm²/hr at 640.75 ± 50.06 nCi/l). Uptake rates were variable and not necessarily dependent upon substrate concentration.

Animal Tissue Extracts

The animal tissue lipid extracts contained 22 autoradiographically visualized metabolites in addition to the parent compound, progesterone. Metabolite distribution varied both within and between time treatments (Table 4.2). Several metabolites were found only in one time point (e.g only in samples from the 3 hour incubation), however, they were often unique to a single colony (n=4). This may be due to intercolony variation rather than the time treatment (Figure 4.4a; Table 4.2). However, two of these metabolites, #2 and #4, are present in both corals from the shorter incubation.

Four metabolites co-migrated with standards. Metabolite #1, #2, #19 and #38 co-migrated with 5-androstenetriol, 11-deoxycortisol, pregnenolone, and 5-androstenediol, respectively. Radiolabelled spots migrating with progesterone and pregnenolone were among the eleven metabolites found in both incubations. All other metabolites did not migrate with known standards. Metabolite 24 could be visualized by UV.

Cholesterol units/cm²

Days After New Moon

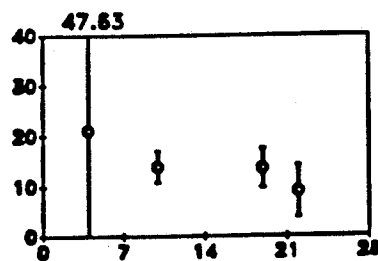
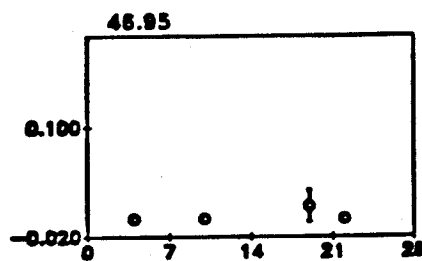
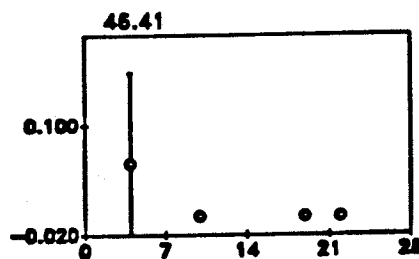
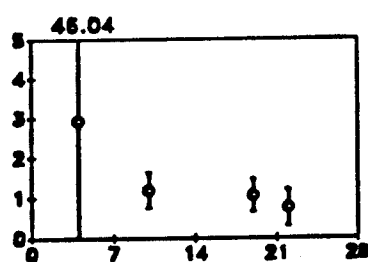
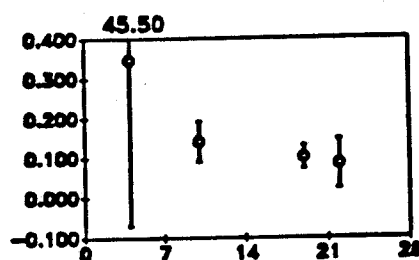
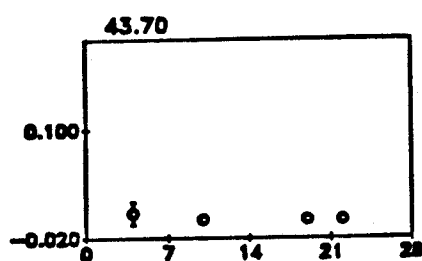
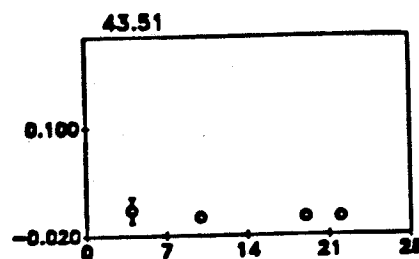
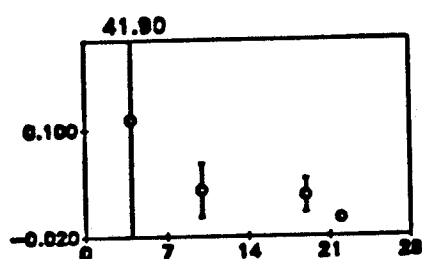
Ten metabolites (29-38) found in the *in vivo* samples were not found in the *in vitro* assays of the four scleractinian species. Eleven *in vivo* metabolites were different from the *in vitro* incubation with *Favia fragum*. Ten *in vitro* metabolites of *F. fragum* were not produced *in vivo*.

Zooxanthellae Extracts

Sixteen metabolites were present in the zooxanthellar lipid extracts but progesterone was not present (Figure 4.4b). Seventy percent of these metabolites (11/16) were present in the host animal extracts. Three zooxanthellar metabolites (#17, #26, #28) were not present in the animal tissue extracts. Metabolite #2, #19 and #38 co-migrated with standards as in the animal tissues.

DISCUSSION

The biochemical pathway from steroid precursors such as cholesterol, pregnenolone, and progesterone to the androgens and estrogens is similar between vertebrate and invertebrate groups (Sandor & Mehdi, 1980), although, unique pathways and end products in invertebrate groups have been demonstrated (Voogt et al., 1990). In this study, scleractinian corals have been shown to perform biochemical transformations of progesterone into many compounds when incubated with animal tissue homogenates and intact coral colonies.

Cholesterol units/cm²

Days After New Moon

Figure 4.4 Diagrammatic representation of two dimensional TLC of radiolabelled metabolites of ^{14}C -progesterone following *in vivo* incubation of the scleractinian coral *Favia fragum*. a) animal tissue extracts from 1.5 hour (n=2) and 3 hour incubation (n=2) b) washed zooxanthellae extracts from 1.5 hour (n=2) and 3 hour incubation (n=2). Metabolites were visualized by autoradiography. Metabolite 28-38 are not found in the *in vitro* assays.

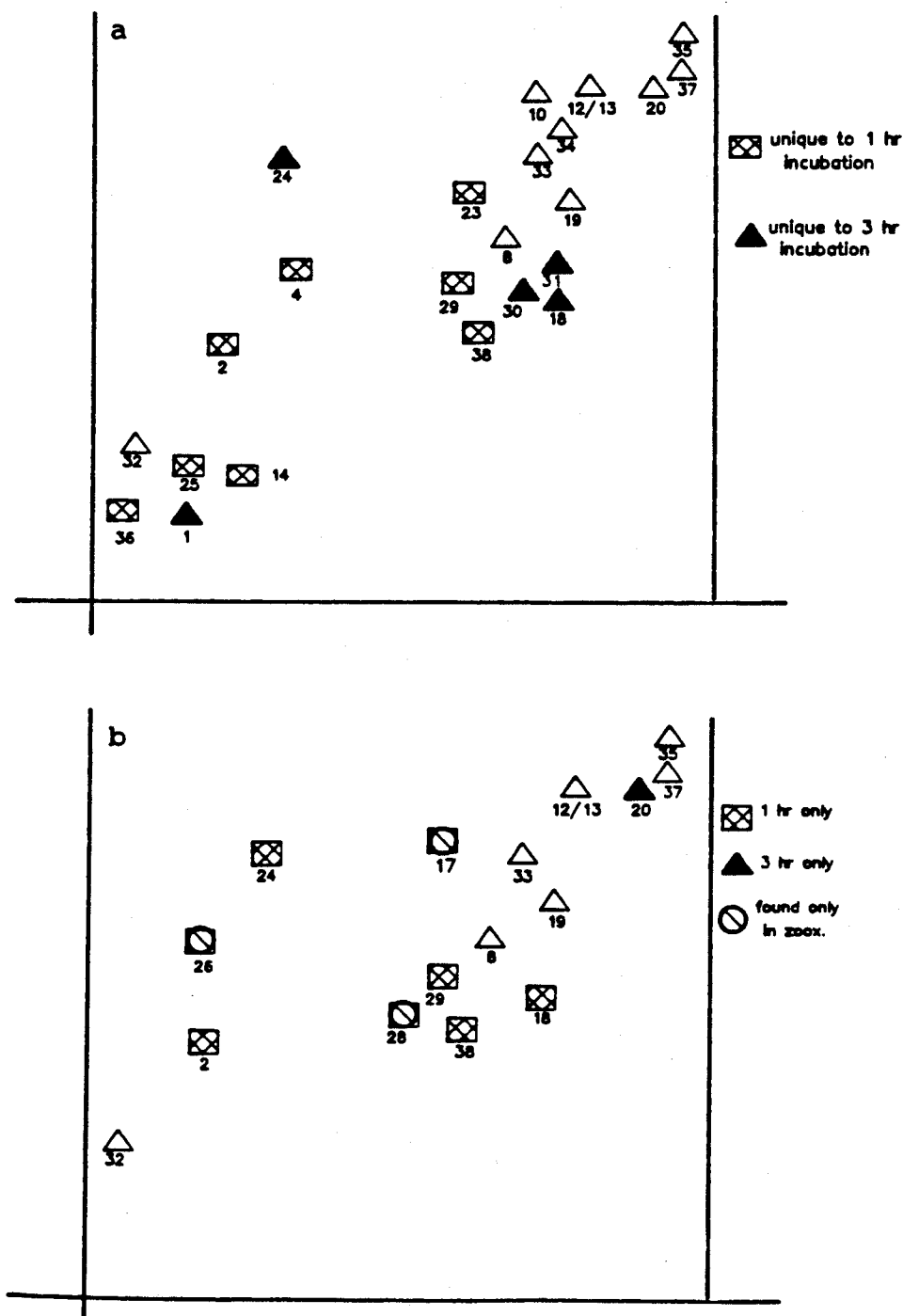
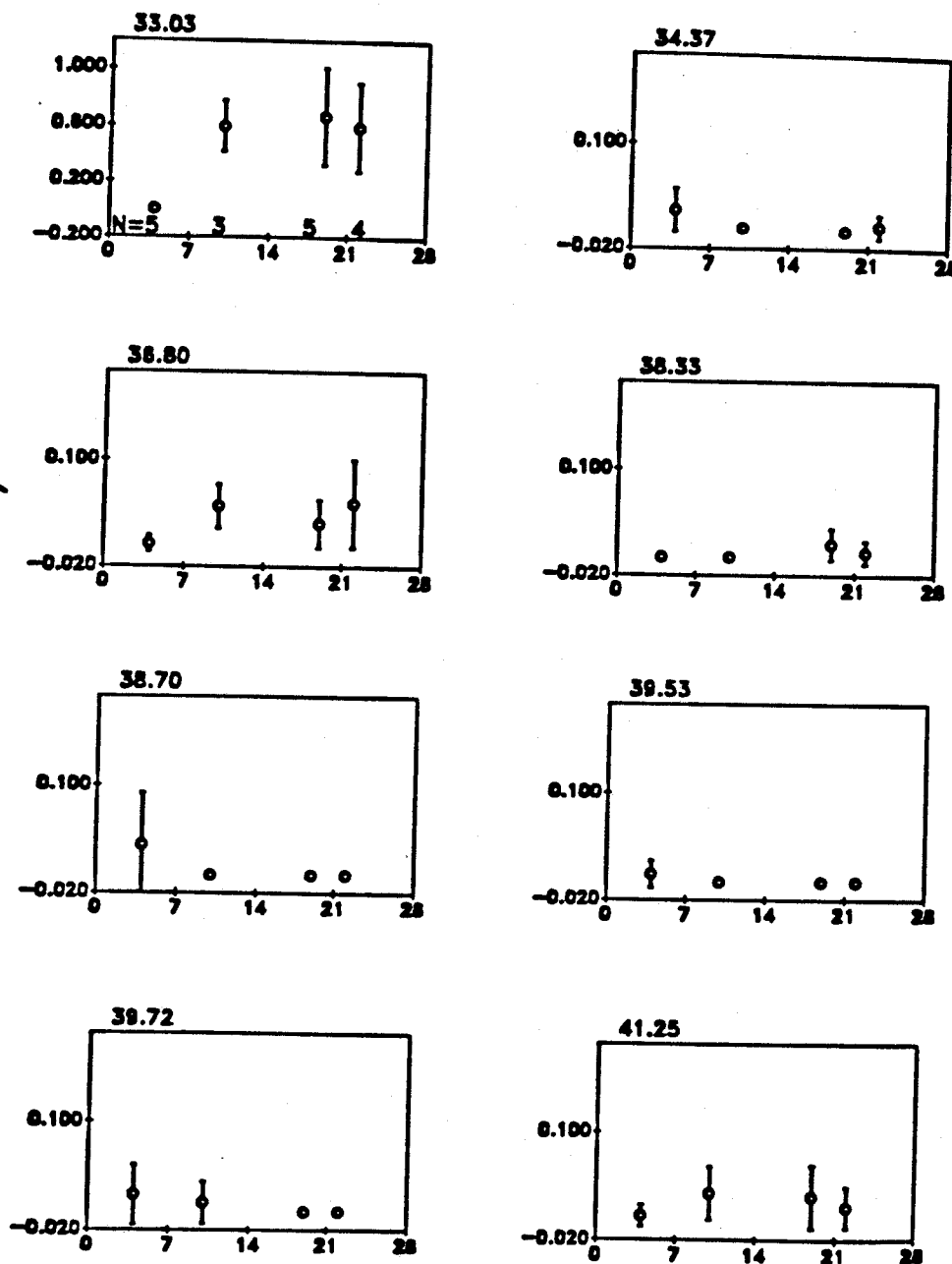


Figure 5.1 1988 *Favia fragum* sampling chromatographic data expressed as cholesterol units per cm^2 surface area. Retention times are listed above each graph. Mean and standard deviation are given with sample size listed in the first graph.

Cholesterol units/ cm^2



Days After New Moon

In vitro incubation

The coral animal tissue contains enzymes which can effect the chemical transformation of progesterone to testosterone. The absence of 4-androstenedione, the expected intermediate compound, from mammalian Δ^4 enzymatic pathways (Figure 1.1) suggests that 1) the reaction equilibrium may lie toward 17α -OH progesterone or testosterone, 2) this intermediate appears and is removed on a time scale shorter than the incubation time of three hours, or 3) a unique enzymatic pathway exists in corals which perform this metabolic transformation. The production of 11-deoxycortisol, a metabolite found in all coral species examined, probably occurred via the pathway through 17α -OH-progesterone. Interestingly, the production of a corticoid has rarely been seen in incubations of this type using invertebrate tissues (Sandor & Mehdi, 1980). The presence of pregnenolone among the metabolites of *Montastrea annularis* and *Favia fragum* suggests that reductive pathways operating in the reverse direction may be present presumably through the enzymatic activity of 3β -steroid dehydrogenase and Δ^5, Δ^4 isomerase. The presence of these enzymatic pathways does not confirm these compounds as specific substrates for the coral animals. However, Swevers et al. (1991) suggest that complex patterns of metabolism of vertebrate-type steroids in invertebrates is suggestive of a physiological role of steroids in the organism. The physiological effect remains to be demonstrated in the

Retention time	RRT	22 ANM	4 ANM	10 ANM	19 ANM	Coeluting standard
		N=5	N=3	N=5	N=4	
49.43	1.038	0	2	0	0	Ergosta-8,22dien-3 β -ol
49.57	1.041	0	0	1	0	
50.08	1.051	0	2	0	0	5 α ergosta-8,22dien-3 β -ol
51.31	1.077	5	3	5	4	
51.77	1.087	5	3	5	4	Ergosta-7,22-dien-3 β -ol
52.17	1.095	0	3	4	3	5 α campenstan-3 β -ol
52.69	1.106	0	2	3	3	5 α campstan-3 β -ol
53.13	1.115	3	3	5	3	
53.33	1.12	1	2	1	2	
53.75	1.128	0	3	4	4	
54.32	1.140	0	0	0	2	
55.32	1.161	0	0	0	1	
55.59	1.167	3	2	5	3	
56.01	1.176	1	3	5	4	Lanosterol
56.72	1.191	5	2	5	4	
56.99	1.197	2	3	5	4	Poriferastan-3 β -ol
58.23	1.223	3	3	5	3	
59.61	1.252	0	0	1	0	
63.88	1.341	5	3	5	4	

cnidarians.

In vivo incubation

Eleven of the 16 metabolites generated by the intact zooxanthellae/ animal symbiotic unit were different from those generated by isolated animal tissue incubations of *Favia fragum*. Testosterone and 17α -OH progesterone were absent in the *in vivo* samples. Reactions *in vitro* may proceed faster due to the addition of NADPH which may exceed natural concentrations. This does not explain the production of 11-deoxycortisol in the absence of 17α -OH progesterone. Incubations using both longer and shorter time periods may better address these differences between *in vivo* and *in vitro* incubations. However, non-biological side-reactions and lack of some reactions in *in vitro* assays cannot be discounted. Finally, differences between the *in vivo* and *in vitro* incubations of *F. fragum* may be due, in part, to the lunar and season cycle of reproduction and the activation of certain enzymatic functions. However, changes in P-450 cytochrome content and related mixed function oxygenases on a lunar and seasonal cycle has not been demonstrated in corals (see Chapter 3), but is seen in other marine invertebrates.

Zooxanthellae extracts had many metabolites found in the host animal but not the parent compound, progesterone and several metabolites were unique to the zooxanthellae. These data suggest that metabolites of progesterone, provided to the

Table 5.2 Retention time of *Favia fragum* sterols in the 1988 sampling. Relative retention time (RRT) to cholesterol is provided. Peaks which share RRT with a standard are listed. Peaks shown here are a composite of all identifiable peaks from the four sampling days. The number of colonies with detectable peaks at each retention time is given. The collection period was 3/22/88 corresponding to 22 days after new moon (ANM) to 4/6/88 (19 ANM).

Retention time	RRT	22 ANM	4 ANM	10 ANM	19 ANM	Coeluting standard
		N=5	N=3	N=5	N=4	
33.03	0.693	5	0	5	4	
34.37	0.722	1	2	1	0	
36.80	0.773	3	1	5	3	
38.33	0.805	1	0	0	2	
38.70	0.813	0	1	0	0	
39.53	0.830	0	1	0	0	
39.72	0.833	0	1	1	0	
41.25	0.866	1	1	3	2	5 α ergosta-8(14)en 3 β -ol
41.90	0.880	0	3	3	3	
43.51	0.913	0	1	0	0	
43.7	0.917	0	1	0	0	
45.5	0.955	4	3	5	4	Cholest-5,22(Z)dien-3 β -ol
46.04	0.967	5	3	5	4	Cholest-5,22(E)dien-3 β -ol
46.41	0.974	0	1	0	0	
46.95	0.986	0	0	0	2	
47.63	1.000	5	3	5	4	Cholesterol
47.79	1.003	4	2	0	1	
47.88	1.005	1	3	5	3	Dihydrocholesterol
48.39	1.016	3	3	5	4	Desmasterol
48.71	1.023	3	2	5	4	
49.15	1.032	5	3	5	4	

zooxanthellae by the host animal, were further metabolized by the algae. This pattern has also been noted in the biochemical transformation of a host sterol, gorgosterol, by the zooxanthellae (Ciereszko et al., 1968). However, further study is needed to completely assess the ability of zooxanthellae to metabolize sterols.

Sterols on the reef

Sterols are biochemical components of both dissolved organic carbon (DOC) and particulate organic carbon (POC) in marine waters. Corals can exploit sources of POC and DOC (Sorokin, 1973), but it has not been previously demonstrated that corals can take up sterols from the water column. The concentrations of total sterols in surface oceanic water range from 0.38-0.90 $\mu\text{g/l}$ seawater in the north Atlantic (Gagosian, 1975). In the eastern equatorial Atlantic waters, the range is somewhat greater (4.0-7.7 $\mu\text{g/l}$ seawater; Saliot & Barbier, 1973). At these concentrations, sterols from marine organisms on the reef are available to the corals. The uptake rate of progesterone in this study (in the concentration range of 2.9 - 4.0 $\mu\text{g/l}$) is similar to that found for the chemically analogous pollutant, benzo[a]pyrene (Kennedy et al., in press) under the same laboratory conditions. Therefore, we predict that corals animals can take up dissolved sterol and sterol-like compounds directly from the marine environment. The corals relatively high lipid content (Patton et al., 1977) and

Table 5.1 Characteristics of 1988 *Favia fragum* samples from preliminary sampling.

CORAL ID	Lunar Day	Surface Area (cm ²)	Total Lipid (mg/cm ²)	Protein (mg/cm ²)	Lipid/Protein
3/11/88 1	22	10.08	0.129	0.575	0.224
2		13.06	0.194	0.670	0.289
3		13.74	0.081	0.328	0.247
4		10.32	0.174	0.674	0.258
5		15.93	0.075	0.334	0.224
3/22/88 1	4	8.54	0.295 **	0.824	
2		13.46		0.937*	
3		19.57	0.252	0.824	0.306
4		8.14	0.286	0.875	0.329
5		9.07			
3/28/88 1	10	9.77	0.281	0.996	0.282
2		12.86	0.300	0.914	0.328
3		11.49	0.225	0.672	0.335
4		15.75	0.154	0.541	0.285
5		15.02	0.236	0.939	0.251
4/6/88 1	19	12.75	0.316	0.980	0.322
2		21.62	--	1.111	
3		22.79	0.229	0.830	0.276
4		13.41	0.329	1.111	0.296
5		9.76	0.232	0.682	0.340

* Sample 2 and 5 were combined before lipid extraction

** Sample 1, 2 and 5 were combined before lipid separation

-- missing data

the lipophilic nature of sterols and some pollutants may facilitate uptake of these compounds by the coral.

CONCLUSION

Metabolism of vertebrate-type steroids by the animal tissues of scleractinian is possible and may follow mammalian enzymatic pathways. Thus, this is the first demonstration on the existence of these pathways in a primitive invertebrates species.

obscures any temporal trends which may be present.

1989 Lunar Sampling

The lunar date, surface area, and protein for the 1989 lunar sampling are listed in Table 5.3. Protein values per surface area fluctuated over the lunar cycle but no significant trends appear when plotted as deviation from the grand mean (Figure 5.4).

Few standards were available for comparison of coelution with samples. With the exception of cholesterol, no peaks were given a tentative identification.

Due to technical difficulties, the internal standard was not used. Repeat injections, problems with the autosampling device, and evaporation of samples prior to injection made normalizing peak area to protein or surface area inaccurate. The data are presented only as percent total peak area (Figure 5.5).

The mean percent total peak area of all corals sampled is compared to that for the planulae sample (Figure 5.6). Two peaks were present in the planulae that were not seen in the coral.

CHAPTER 5

MONTHLY VARIATION IN THE STEROL CONTENT OF THE SCLERACTINIAN CORAL, FAVIA FRAGUM.

INTRODUCTION

Steroids which regulate reproductive cycles are well known in mammalian systems and can be found in invertebrate systems as well. The ecdysteroids found in Arthropods are one example of a specific class of steroids which regulate a reproductive function in invertebrate groups (Sandor, 1980). The prevalence of steroids in cnidarian tissue extracts coupled with the early phylogenetic occurrence of distinct gonads in this group, make cnidarians likely candidates for investigation into the evolution of steroids as biochemical regulators of reproduction.

Reef cnidarians, gorgonians and alcyonaceans in particular, contain many unusual sterols. While many of these compounds have been identified, little or no attention has been given to the role sterols may play in the physiology of the organism. In the gorgonian *Muricea californica*, sterols have been shown to vary seasonally (Popov et al., 1983). In the zooanthid *Palythoa caribaerum*, gorgosterol content was noted to be different between genders (Kelecom & Solé-Cava, 1982). It has

pregnenolone, and estrone.

Data from each date was compiled and the four sampling dates compared. Samples from 3/11/88 (22 ANM) had the fewest peaks with 21 and 3/22/88 (4 ANM) the most with 33. Only four peaks were present in all sample chromatograms (Table 5.2). Eight peaks were found only in the extracts of a single colony of the 17 analyzed. In addition, four peaks appeared only within a single collection date (retention times = 46.95, 49.43, 50.08, and 54.32). Multiple zero values and violation of the assumption of each sample being an individual from a unique population (i.e. on 3/22/88, the lipids of 3 colonies were mixed prior to chromatographic analysis) made statistical analysis difficult. Therefore discussion of the results will be limited to qualitative observations.

Individual peaks expressed as cholesterol units were normalized to living surface area (Figure 5.1), and normalized to supernatant protein (Figure 5.2). Data were also expressed as percent of the total peak area of the chromatogram to reflect a compound's contribution relative to the total sterols present (Figure 5.3).

The sterol profile of each of the four sampling dates was very similar when normalized to surface area or protein. The percent of the total sterol peak area appears to be the least variable of the data normalizations attempted. In general, the samples from 3/22/88 were the most highly variable. Additionally, individual variation within each date sampled

been suggested that seasonal synthesis of sterols may indicate a role in the annual reproductive cycle in these organisms. This is seen in the anemone, *Metridium senile*, where the percent of sterols rose from 35% to 60% of the neutral lipids during gametogenesis (Hill-Manning & Blanquet, 1979).

Favia fragum, a small scleractinian coral, has a lunar cycle of gametogenesis, planulation and spawning (Szmant-Froelich et al., 1985). Spermatogenesis is begun and completed in a single lunar cycle. While oogenesis is continuous, only one or two oocytes per mesentery mature per cycle. Steroids may play a role in the biochemical regulation of these predictable cycles. The current investigation examines the sterol content over a monthly cycle of reproduction in *F. fragum*.

MATERIAL AND METHODS

Two series of collections were made (1) March 11 to April 6, 1988 and (2) October 25 to December 9, 1989. Different sample preparation techniques were used for each collection and thus each is described separately.

Collection and tissue processing for 1988 sampling

Five colonies of *Favia fragum* were collected approximately once a week for four weeks from Fowey Rocks Lighthouse, FL (25°35.4'N, 80°5.8' W) in March and April, 1988. Colonies were

Integrated peak areas were used from retention times in the range of the steroid standards to the end of the run (33 min to 80 min). Only those chromatograms showing a cholesterol peak at 62.41 were analyzed. The data were graphed as percent total peak area.

RESULTS

1988 Sampling

Surface area, total lipid and protein for the 1988 sampling is listed in Table 5.1. Mean lipid and protein content was significantly different over the sampling time (ANOVA, $p < 0.006$) with the greatest values occurring on the last day (April 6, 1988). Multiple comparison analysis showed lipid and protein content of the first sampling date (3/11/88) to be different from that of the other sampling dates (Tukeys, lipids, $p < 0.023$; protein, $p < 0.059$).

A total of 40 peaks eluted from 33.03 to 63.88 min retention time (raw data in appendix). Of these, thirteen coeluted with known standards (Table 5.2). However, these standards were not co-injected with samples. Cholesterol was the dominant sterol comprising more than 40% of the total peak area, equalling 3.9-47.3 $\mu\text{g}/\text{cm}^2$ surface area. Seven of the 40 compounds eluted in the range of the mammalian steroid standards but none of these coeluted with the standards used which included derivitized progesterone, testosterone,

removed from the substrate with a hammer and chisel at 2-3 m depth while snorkeling.

The first collection date (3/11/88, 22 days after new moon (ANM)) corresponded to the initiation of maturation of oocytes as described in Chapter 2. 3/22/88 (4 ANM) corresponded to initiation of maturation of spermaries. 3/28/88 (10 ANM) corresponded to a peak period of planulation. The final collection date, 4/6/88 (19 ANM), corresponded to several days following spawning of gametes and the first appearance of embryos.

Tissues were removed from the coral skeleton with pressurized air and 0.45 μ m filtered seawater from an artist's airbrush (65 p.s.i.) and kept on ice throughout the procedure. The coral tissue was homogenized with an Ultra-Turrex for 30 s, centrifuged for 4 min at 2000xg at 4°C in a Sorval RC5B superspeed centrifuge and the pellet, containing zooxanthellae, skeletal remnants and larger animal fragments, was discarded. Living tissue surface area was estimated using the aluminum foil method of Marsh (1970).

Extraction

Total lipids were extracted in CHCl_3 :MeOH:H₂O (Bligh & Dyer, 1959) overnight and allowed to phase partition for several hours. The aqueous layer was removed and the remaining solvent was filtered through Whatman 2V folded filter paper and rotary evaporated. Lipid residues were transferred

1989 Lunar collection

To generate a more complete picture of changes in the sterol profile over the lunar period, colonies of *Favia fragum* were collected every 3-4 d from October 25 to December 9, 1989, from several reefs near Alina's reef (25°23.25'N, 80°09.8'W) in Biscayne National Park, FL using the methods above. Tissues were extracted upon return to the laboratory the same day. Corals were airbrushed, homogenized and centrifuged as above. Eighty planula larvae of *Favia fragum* were obtained from corals on November 6, 1989 (8 ANM) and processed identically to the corals.

Extraction

The animal tissue homogenates were extracted using continuous agitation with equal volumes of dry diethylether for 5 min, quick frozen at -45°C in an MeOH bath, and the ether poured into a CHCl₃-washed test tube. The homogenates were extracted twice. Lipid residues were dried under N₂ at 35°C, and stored at -18°C until analysis.

Gas chromatography

Lipids were separated and sterols derivitized as above. The thermal program was run to 280°C and helium was used as the carrier gas at a flow rate of 25 cm/sec. TMS-nortestosterone was added as an internal standard.

quantitatively to a test tube, dried under N_2 at $35^\circ C$, and stored at $-4^\circ C$ until analysis. Samples were stored for no longer than one month. An aliquot of supernatant was removed prior to extraction to quantify soluble protein by the Lowry technique using bovine serum albumin fraction V standard (Lowry et al., 1951). Lipids were quantified gravimetrically in 2 cm tall glass vials on a Sartorius balance.

Separation of lipids and sterols

The lipid residue from each colony was dissolved in $CHCl_3$ and loaded quantitatively onto a disposable silica gel column (JT Baker 3 ml silica gel columns, 7086-3) conditioned with GC/MS grade MeOH (5ml) and $CHCl_3$ (6 ml). The neutral lipid fraction, containing the sterols, was eluted with 5 ml $CHCl_3$, dried under N_2 at $35^\circ C$, and loaded onto preconditioned diol columns (JT Baker 3 ml diol columns, 7094-3) with 80:20 GC:MS grade hexane:diethylether. The columns were vacuum dried for 3 min and then wetted with 92:8 hexane:propanol. Sterols were eluted off the column with additional 92:8 hexane:propanol. Sterol residues were derivitized with 2:1 silylation grade pyridine:bis(trimethylsilyl)trifluoroacetamide (BTSFA, Sigma Chemical Co.) for 15 minutes at $60^\circ C$, cooled, dried over N_2 , and dissolved in one ml hexane. This formed trimethylsilyl (TMS) ethers of hydroxyl and carbonyl groups.

Gas chromatography

One μ l samples were autoinjected using a 19405A Hewlett-Packard sample control module into a 60 m DB-1 capillary column (0.25 μ J&W Scientific) in a Hewlett-Packard 5890A gas chromatograph, temperature programmed for 130-275°C at 4°C/min with hydrogen as the carrier gas. Injector and detector ports were set at 275 and 280°C, respectively. A 3390A HP integrator was connected to the flame ionization detector. Extraction, column, and solvent controls were run at the same time.

Data analysis

All peaks eluting after 30 minutes were chosen for analysis. This time frame begins just prior to the elution of the smallest molecular weight steroid standards. Relative retention times (RRT) for each steroid were calculated using cholesterol (retention time = 47.63 min) as the reference. RRT were compared with known standards. Several steroids were tentatively identified by coelution with authentic standards. Weight expressed as cholesterol units (ug) per surface area, and as cholesterol units per mg supernatant protein using cholesterol standard to estimate ug/peak area, as well as percent total sterol were calculated from peak integration. The expression of weight as cholesterol units will underestimate and overestimate the true contributions of some compounds due to the variable response of the flame ionization detectors to different molecules.