Temporal Synergism of Prolactin and Adrenal Steroids in the Regulation of Fat Stores[1] (35589)

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Seasonal fluctuations and developmental changes in body fat stores are common among vertebrates. Seasonal fluctuations often reflect changes in the environment and serve as adaptive requirements for other physiological events, such as reproduction. Developmental changes also occur in a maturing animal. Inasmuch as a high degree of organization is necessary to coordinate fattening with physiological events, as well as with environmental changes, it seems probable that regulatory systems are involved in controlling levels of fat storage.

Our studies indicate that prolactin, a hormone found in the adenohypophysis of all the major vertebrate classes, has marked effects on the levels of fat stores. In 1 week of daily injections, prolactin elicits gains of 75 to 400% in fish (1, 2), frogs (2), lizards (2), and birds (3). However, the time of day when the injections are made is of singular significance. On 16-hr daily photoperiods, injections at midday elicit fattening; whereas, injections made early in the day result in losses of fat stores.

Daily variations in the fattening responses to prolactin suggest that another system sets the time of tissue responses to prolactin. In a fresh water killifish, *Fundulus chrysotus*, this system is affected by thyroxin injections (4). On continuous light, thyroxin injection sets up a period of fattening sensitivity 18 hr later, a period of sensitivity that recycles every 24 hr with no additional injections of thyroxin. Fattening results when prolactin is given daily during the sensitive period. It was concluded that thyroxin injections set the biological time clock of a system capable of circadian oscillations independent of the photoperiod, a system that sets the temporal pattern of fattening responses to prolactin.

The number of systems that may be expected to regulate rhythms at the tissue or organismal level is limited. Because the adrenocortical hormones have pervasive influences throughout the vertebrate body, and because the plasma levels exhibit circadian oscillations that are synchronized by the photoperiod (5), it seems possible that a daily rhythm of plasma adrenocortical hormones may phase or drive rhythms of fattening sensitivity to prolactin. The experiments reported herein were designed to test whether the interval between daily injections of prolactin and of adrenocortical hormones is important with respect to the levels of body fat stores in representatives of several vertebrate classes.

Materials and Methods. Experiments were performed on three vertebrate species, a teleost fish, *Fundulus grandis*, a lizard, *Anolis carolinensis*, and a pigeon, *Columba livia*. To remove photoperiodic cues to the animal that could render difficult the interpretations of the results, the studies were performed in continuous bright light (fluorescent). The fish and pigeons were given an overabundance of commercial fish and pigeon feed, respectively. The lizards were fed crickets and meal worms *ad libitum*. In three of the experiments, the animals were divided into two groups receiving daily injections of an adrenal steroid hormone; one group received the hormone at 0600 hr and the other re-

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TABLE I. Temporal Synergism of Hydrocortisone and Prolactin in the Regulation of Body Fat and Liver Fat in *F. grandis*.[a]

Hydrocortisone/ saline injection	Time	Measurement of fat in	Responses to prolactin injections (hr after hydrocortisone/saline) 0	6	12	18	Means
Hydrocortisone	0600	Body	5.73 ± 0.26	4.45 ± 0.25	4.71 ± 0.31	6.25 ± 0.50	5.28
		Liver	42.54 ± 1.16	36.16 ± 1.05	40.75 ± 1.27	44.17 ± 1.26	40.90
Hydrocortisone	1800	Body	5.80 ± 0.58	4.02 ± 0.27	4.76 ± 0.39	6.52 ± 0.41	5.28
		Liver	44.46 ± 1.42	39.48 ± 1.08	42.32 ± 1.63	45.70 ± 1.10	42.99
Saline	0600	Body	6.40 ± 0.79	6.36 ± 0.49	6.16 ± 0.52	6.39 ± 0.71	6.33
		Liver	41.65 ± 0.95	39.98 ± 1.14	41.35 ± 1.07	40.71 ± 0.97	40.92

Least squares analysis of variance[b]

	Source	*df*	Sum of squares	Mean squares	*F*	Probabilities
Body fat	Total	60	2008.964			
	Treatment (vs saline)	2	14.484	7.242	5.838	.05
	Time	3	20.334	6.778	5.464	.05
	Linear 1		1.580	1.580	1.274	NS
	Quadratic 1		18.626	18.626	15.016	.01
	Cubic 1		0.128	0.128	0.104	NS
	Treat. × time	6	9.299	1.550	1.249	NS
	Error	48	59.541	1.240		
Liver fat	Total	59				
	Treatment (vs saline)	2	61.657	30.829	4.394	.05
	Time	3	217.706	72.568	10.344	.01
	Linear 1		15.232	15.232	2.171	NS
	Quadratic 1		148.964	148.964	21.234	.01
	Cubic 1		53.510	53.510	7.628	.01
	Treat. × time	6	75.244	12.541	1.788	NS
	Error	48	336.734	7.015		

[a] The fish in groups of five were placed in 10% seawater on continuous light and 24°, March 30, 1970. Injections of hydrocortisone (2 μg/g of body wt/day) were initiated on April 17 and continued daily for 7 days. Six daily injections of prolactin (1 μg/g of body wt/day) were begun on April 18. The fish were killed on April 24. The livers were removed, dried, weighed, and the fat was extracted with petroleum ether using Soxhlet apparatus. The liver fat is expressed as percentage of dry liver weight. The carcasses were treated similarly and the results are expressed as percentage of dry body wt.

[b] The statistics are shown for the hydrocortisone-treated fish. There are no temporal differences in responses to prolactin among the saline-treated groups.

ceived the hormone 12 hr later at 1800 hr. Both groups receiving steroid hormone were further divided into four subgroups receiving daily injections of prolactin at 0600, 1200, 1800, or 2400 hr. This method allowed us to compare 2 groups receiving daily prolactin injections at 0, 6, 12, or 18 hr after adrenal steroid treatment and provided a simple means of determining whether the variations in the responses to prolactin were set by the time of injection of the adrenocortical hormone. In another experiment using the Gulf killifish, hydrocortisone injections were made on 2 days prior to the injections of prolactin but not during the period (June 23–29) when the prolactin injections were made. Injections in the above experiments were given intraperitoneally in the fish, subcutaneously in the lizard, and intramuscularly in the pigeon.

The tables containing the results may be consulted for further information concerning the regimen of the individual experiments.

Results. Two experiments were performed on the brackish water Gulf killifish, *F. grandis*. In one experiment prolactin and hydrocortisone, the principal adrenal steroid in killifish (6), were injected daily in various time relations for 6 days. The highest levels of liver and body fat were found in the groups receiving prolactin 18 hr after hydrocortisone (Table I). The lowest levels were found in the groups receiving prolactin 6 hr after hydrocortisone. Although the shapes of the curves were similar for the 2 groups receiving hydrocortisone at 0600 and 1800, the levels of liver fat were greater in the group receiving hydrocortisone at 1800. In general, hydrocortisone lowers the body fat but not the liver fat in prolactin-treated fish. No daily variations were found in the prolactin-treated fish receiving saline injections rather than hydrocortisone. Statistical evaluations are provided in Table I.

In a second experiment using *F. grandis*, hydrocortisone was injected twice only (19 and 21 June), either at 0600 or at 1800. Prolactin was injected daily for 7 days at one of 4 different times (0600, 1200, 1800, or 2400) beginning on 23 June. Pretreatment with hydrocortisone set up a persistent rhythm of fat responsiveness to prolactin (Table II). Peak levels of liver and body fat stores were found in fish receiving prolactin 18 or 24 hr after the time of hydrocortisone pretreatment, whereas the lowest levels of fat stores were found in fish receiving prolactin 6 and 12 hr after the pretreatment time. Although only the daily variations in liver fat responses were valid statistically (see Table

TABLE II. Circadian Responses to Prolactin in *F. grandis* after Treatment with Hydrocortisone.[a]

Hydrocortisone/ saline injection (June 19 and 21)	Time	Measurement of fat in	Responses to prolactin injections (June 23–29) (hr after time of hydrocortisone/saline pretreatment) 0	6	12	18	Means
Hydrocortisone	0600	Body	4.42 ± 0.42	3.01 ± 0.52	3.72 ± 0.59	4.36 ± 0.35	3.88
		Liver	39.38 ± 1.27	35.01 ± 2.07	31.02 ± 1.08	36.98 ± 1.46	35.60
Hydrocortisone	1800	Body	4.35 ± 0.41	3.32 ± 0.47	3.81 ± 0.37	5.26 ± 0.60	4.19
		Liver	41.17 ± 2.46	31.61 ± 3.18	31.24 ± 3.22	39.03 ± 2.25	35.76
Saline	0600	Body	4.61 ± 0.57	4.12 ± 0.92	4.08 ± 0.64	4.87 ± 0.61	4.42
		Liver	32.66 ± 2.35	33.47 ± 2.72	30.98 ± 2.36	31.81 ± 2.47	32.26

Least squares analysis of variance[b]

	Source	*df*	Sum of squares	Mean squares	*F*	Probabilities
Liver fat	Total	65				
	Treatment	2	129.193	64.596	2.176	NS
	Time	3	385.911	128.637	4.334	.05
	Linear 1		53.438	53.438	1.800	NS
	Quadratic 1		292.538	292.538	9.856	.01
	Cubic 1		39.935	39.935	1.346	NS
	Treat. × time	6	295.551	49.258	1.660	NS
	Error	54	1602.738	29.680		

[a] The fish in groups of five or six were placed in continuous light and 24° on June 8, 1970. Injections of hydrocortisone (2 μg/g of body wt/day) were made on June 19 and 21. Daily injections of prolactin (1 μg/g of body wt/day) were made June 23–29. The fish were killed June 30 for body fat (% dry body wt) and liver fat (% dry liver wt) determinations.

[b] The statistics are shown for the responses of liver fat to prolactin in the hydrocortisone-treated fish. Body fat responses were statistically analyzed and the time differences were found not to be significant at the 95% confidence levels. Among the saline-treated groups, there are no temporal differences in responses to prolactin.

TABLE III. Temporal Synergism of Corticosterone and Prolactin in the Regulation of Fat Body Weights (% body wt) in *A. carolinensis*.[a]

Corticosterone/ saline injections	Time	Responses to prolactin (hr after corticosterone/saline) 0	6	12	18	Mean
Corticosterone	0600	2.06 ± 0.14	1.30 ± 0.10	1.54 ± 0.18	2.20 ± 0.34	1.78
Corticosterone	1800	2.62 ± 0.46	1.52 ± 0.18	2.46 ± 0.22	2.01 ± 0.20	2.15
Saline	1800			1.00 ± 0.17		

Least squares analysis of variance

Source		*df*	Sum of squares	Mean squares	*F*	Probabilities
Total		51				
Treatment (C_{0600} vs C_{1800})	1		1.839	1.839	5.225	.05
Time	3		5.438	1.813	5.149	.01
Linear	1		0.018	0.018	0.051	NS
Quadratic	1		2.925	2.925	8.308	.01
Cubic	1		2.496	2.496	7.089	.05
Treat. × time	3		2.301	0.787	2.235	NS
Error		44	15.490	0.352		

[a] The lizards were placed in continuous light and 30 ± 2° on May 7, 1970. Injections of corticosterone were initiated on May 22 and of prolactin on May 27. Daily injections were continued until June 5 when all the animals were killed and the fat bodies weighed. Each group had 6 or 7 lizards. One group at the beginning of hormone treatment had fat bodies that were 0.87 ± 0.23% fresh body wt. Another untreated group at the end of the hormone treatment had fat bodies that were 1.70 ± 0.23% fresh body wt.

II), the similarity of the daily rhythm of body fat responses suggests that it also is phased by the time of hydrocortisone pretreatment. Pretreatment with saline did not phase rhythms of fat responses to prolactin.

One experiment was performed on green anoles, *A. carolinensis*, which were maintained at 30 ± 2°, their preferred temperature (7). Daily injections of prolactin were made for 7 days at several time intervals (0, 6, 12, or 18 hr) following the daily injections of corticosterone, the principal adrenal steroid in some lizards (8). The weight of the abdominal fat body was used as the index of fat responses to the hormones. The weights of the fat bodies were lowest in the groups receiving prolactin 6 hr after corticosterone regardless of whether the adrenal steroid was given at 0600 or at 1800 (Table III). The highest weights centered on the groups receiving prolactin 18 to 24 hr after corticosterone. When compared with the fat bodies of untreated lizards before (0.087% body wt) and after (1.70% body wt) the experiment, as well as with those of a saline-injected group (1.00% body wt) the fat bodies were enlarged in those lizards receiving prolactin 18 (2.10% body wt) and 24 (2.34% body wt) hr after corticosterone injections.

In the common pigeon, *C. livia*, prolactin injections were made daily for only 4 days. Nevertheless, several rhythms of tissue responsiveness to prolactin were phased by corticosterone, the principal adrenal steroid in birds (9). Inasmuch as marked daily variations in cropsac responses to prolactin have also been found in the pigeon [(10), and Burns and Meier, in preparation] we weighed the dry mucosal scrapings [for procedure (11)] to determine whether corticosterone could phase a rhythm of cropsac sensitivity as well as rhythms of liver and abdominal fat and of intestinal weight responses to prolactin. Although only the rhythms of the intestinal weight and of the cropsac responses are valid statistically (Ta-

TABLE IV. Temporal Synergism of Corticosterone and Prolactin in the Regulation of Organ Weights in the Pigeon, *C. livia*.[a]

Corticosterone/ saline injection	Time	Measurement in	Responses to prolactin injections (hr after corticosterone/saline) 0	6	12	18	Means
Corticosterone	0600	Abdominal fat	7.7 ± 1.3	6.0 ± 0.8	6.9 ± 0.7	5.3 ± 0.4	6.5
		Liver fat	18.6 ± 4.2	11.1 ± 1.0	13.6 ± 1.4	11.6 ± 0.5	13.7
		Intestine wt	13.7 ± 1.4	11.3 ± 1.3	11.4 ± 0.5	11.5 ± 0.4	12.0
		Cropsac mucosa	36.5 ± 6.5	36.5 ± 7.0	27.7 ± 4.2	43.7 ± 6.8	36.1
Corticosterone	1800	Abdominal fat	7.1 ± 0.9	4.4 ± 0.8	5.6 ± 1.2	6.1 ± 1.1	5.8
		Liver fat	13.1 ± 1.8	11.7 ± 1.9	12.2 ± 1.8	13.3 ± 1.5	12.6
		Intestine wt	16.5 ± 1.6	9.6 ± 0.6	11.7 ± 1.4	11.0 ± 0.5	12.2
		Cropsac mucosa	51.0 ± 6.3	38.0 ± 9.4	34.4 ± 2.8	62.3 ± 9.3	46.4
Saline	0600	Abdominal fat	7.7	10.8	9.5	9.0	9.3
		Liver fat	12.5	12.9	13.7	15.5	13.6
		Intestine wt	16.4	20.0	13.0	12.6	15.5
		Cropsac mucosa	65.9	72.4	68.6	57.2	66.1

Least squares analysis of variance[b]

	Source		*df*	Sum of squares	Mean squares	*F*	Probabilities
Intestine wt	Total		46	7132.360			
	Treatment	1		0.677	0.677	0.103	NS
	Time	3		140.917	46.972	7.126	.01
	Linear	1		60.308	60.308	9.149	.01
	Quadratic	1		51.560	51.560	7.822	.01
	Cubic	1		29.050	29.050	4.407	.05
	Treat. × time	3		31.245	10.415	1.580	NS
	Error		38	250.492	6.592		
Cropsac mucosa	Total		46	94818.600			
	Treatment	1		1210.496	1210.496	4.243	.05
	Time	3		3155.319	1051.773	3.687	.05
	Linear	1		302.006	302.006	1.059	NS
	Quadratic	1		2409.570	2409.570	8.447	.01
	Cubic	1		443.743	443.743	1.556	NS
	Treat. × time	3		507.472	169.157	0.593	NS
	Error		38	10840.123	285.266		

[a] The pigeons were placed in continuous light and 25° on April 2, 1970. Injections of corticosterone (300 μg/day) were initiated April 16 and continued for 6 days. Prolactin injections (150 μg/day) were made for 4 days beginning April 18. The pigeons were killed April 22; and the abdominal fat pad and intestines were weighed. Liver fat was extracted by petroleum ether using Soxhlet apparatus and expressed as percentage of dry wt. The cropsac was removed and a 4-cm disc of mucosa was removed, dried, and weighed. Each of the corticosterone-treated groups were composed of 4 subgroups of 5 or 6 pigeons. The groups receiving no corticosterone were composed of 3 pigeons each.

[b] The statistics were performed on the corticosterone-treated birds.

ble IV), the responses of the liver fat and the abdominal fat are similar to that of the intestinal weight and are judged to be phased also by corticosterone. The similarities in the phases of the response rhythms of the intestinal weight, liver fat, and abdominal fat are evident in Fig. 1. The cropsac response rhythm, however, is not in phase with these 3 rhythms. Whereas the peak cropsac response occurs 18 hr after corticosterone, the peak responses of the liver and abdominal fat and of the intestinal weight occur 24 (or 0) hr

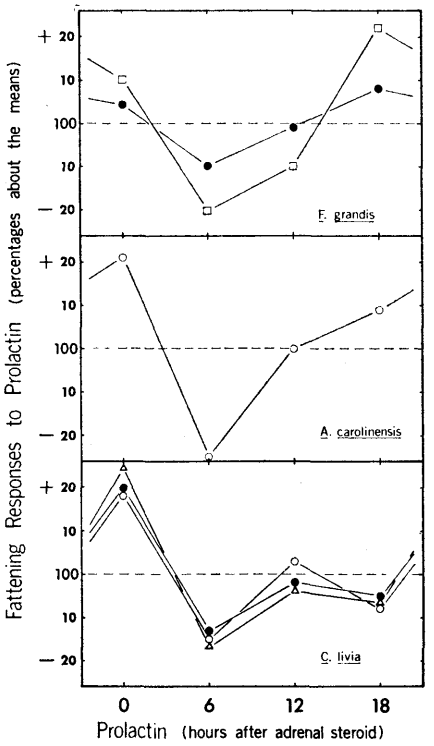

FIG. 1. Daily variations of fattening responses to prolactin phased by adrenal steroids. The graph was compiled from data shown in Tables I, III, and IV. Symbols: (■) body fat; (●) liver fat; (○) abdominal fat body (*A. carolinensis*) or abdominal fat pad (*C. livia*); (△) intestine weight.

after corticosterone. In addition, the least response of the cropsac occurs 12 hr after corticosterone, and the lowest fat levels and intestinal weights occur in the groups receiving prolactin 6 hr after corticosterone. With the exception of liver fat, corticosterone has an overall suppressing effect on the organ and tissue weights when compared with the saline-injected controls.

Discussion. The results indicate that storage levels of body fat may be controlled in several vertebrates by a temporal synergism of prolactin and adrenal steroids. Adrenocortical hormones appear to phase or drive 24-hr oscillations of tissue sensitivity to prolactin. The interval of time between the daily injections of the hormones determines the level of fat stores. In general, the combination which favors low levels of fat is that in which prolactin follows the adrenal steroid by 6 hr. High levels of fat stores result from the injections of prolactin 18 to 24 hr following an adrenal steroid.

Interpretations of experiments involving injections of prolactin and adrenal steroids should take into account the possibility of interaction of the exogenous hormones with endogenous hormones. It seems probable that the daily variations in fat responses to injected prolactin (1–3) are a result of oscillations of tissue sensitivity phased or driven by circadian fluctuations of plasma adrenocortical hormones. Similarly, daily variations in fat responses to injections of adrenocortical hormones (Meier and Martin, in preparation) may depend on the periodic release of pituitary prolactin.

Because many circadian rhythms tend to dampen out under conditions of continuous bright light, an effort was made to reduce temporal interference from the endogenous system by eliminating photoperiodic synchronization of the hormone rhythms. However, endogenous hormone rhythms are still discernible in some of the experiments performed in continuous light. For instance, in the experiment with lizards (Table III), the fat bodies were heavier in the groups receiving corticosterone at 1800 than at 0600. It appears that daily rhythms of endogenous prolactin were in synchrony among the experimental animals, and that the time of injection of the adrenal steroid with respect to the release of endogenous prolactin was more favorable for fattening at 1800 than it was at 0600. Similar rationale may explain the differences in cropsac mucosal weights of pigeons receiving corticosterone at 2 times of day (Table IV). Moreover, if we assume that fat animals have a temporal pattern of adrenal steroids and prolactin that favors higher levels of fat stores and that lean animals have a temporal synergism of hormones that favors lower levels of body fat, it follows that the injections of either hormone would alter the temporal pattern of the endogenous system and probably result in loss of fat in the fat animals and gain of fat in the lean animals. These results were obtained in the experiments with the lizards and the pigeons. The lizards used in the experiment were lean

relative to the levels found in lizards at other times of the year. Combinations of the exogenous hormones caused fat gains in the relatively lean lizards. The young pigeons that were used were relatively fat compared with older pigeons; and the exogenous hormones tended to reduce the levels of fat stores.

In a previous experiment on a fresh water fish, *F. chrysotus*, thyroxin injections initiated a self-sustaining circadian oscillation of fattening responsiveness to prolactin (4). It was concluded that thyroxin sets the biological time clock of another system capable of sustained oscillations independent of photoperiodic information. The experiments reported herein suggest further that the adrenocortical (interrenal) system phases or drives the tissue responses to prolactin, and that it is capable of self-sustaining oscillations which may be synchronized initially by injections of adrenal steroids (Table II). We believe that the exogenous steroids resynchronize the endogenous ACTH-adrenal system by way of a negative feedback on the hypothalamo-hypophyseal system. The existence of a self-sustaining oscillation of sensitivity to prolactin that can be timed by injections of adrenal steroids constitutes strong evidence that circadian systems are involved in the control of fat levels.

Although our results are consistent with the hypothesis that thyroxin injections phase rhythms of fattening responses to prolactin in fish by phasing the interrenal system, it is also possible that thyroxin and adrenal steroids act independently on the tissues involved in lipid metabolism. In pigeons, thyroxin does not appear to phase rhythms of fattening and cropsac responses to prolactin, but it does influence the shape and the length of the period of free-running rhythms under continuous light [(12) and John and Meier, in preparation].

The role of daily rhythms of prolactin in the control of seasonal levels of fat stores has been studied most extensively in the white-throated sparrow, *Zonotrichia albicollis*. In fat sparrows during the vernal migratory period, pituitary prolactin is released during the afternoon (13), a time of day when prolactin injections can elicit gains of fat stores in lean birds (3). Moreover, in lean birds in midsummer, pituitary prolactin is released during the dark shortly before dawn (13), a time when injections of prolactin cause losses in fat stores (3). Daily rhythms of prolactin have also been reported in the laboratory rat (14) and the hamster (15), but they were not correlated with any physiological condition.

Daily rhythms of adrenal steroids have received much more attention than those of prolactin (5). Rhythms have been described in representatives of most of the major vertebrate taxa. The rhythms of the adrenal steroids have been correlated with other daily rhythms and are thought to drive many of them. In *Z. albicollis*, daily rhythms of plasma corticosterone were found to differ seasonally (16). For each of the 4 seasons when the birds were in 4 distinct physiological conditions, there was a specific rhythm of plasma adrenal steroids having individual phase angles with respect to the photoperiod. In further support of the hypothesis that the daily rhythms of adrenal steroids have a role in the regulation of seasonal levels of body fat, we have recently discovered that there are also daily fattening responses in the white-throated sparrow to injections of corticosterone as well as to prolactin (Meier and Martin, in preparation).

Although migratory fattening is not involved in the animals reported herein, seasonal and developmental changes in fat stores do occur. In green anoles, seasonal changes in body fat levels have been associated with the requirements of overwintering and reproduction (17). Fat levels in the pigeon are associated with age, younger pigeons being fatter than mature pigeons (18). Both reproduction and age are associated with fat levels in fish (19).

The regulation of fat storage would seem to require organization of many metabolic processes and systems. The daily variations in responses to prolactin of several tissues in the pigeon seem of special interest with regard to temporal organization. The greatest stimulatory effect of prolactin on liver fat, abdominal fat, and intestine weight in all instances occurred at the same time interval following corticosterone injection (about 24

hr); whereas the greatest stimulatory effect of prolactin on the cropsac occurred at another time (18 hr after corticosterone). Thus, the temporal pattern of corticosterone and prolactin that favors fattening also favors splanchnomegaly, but it does not favor proliferation of the cropsac and the production of pigeon "milk." Such a system allows one hormone, such as prolactin or an adrenal steroid, to be involved in the regulation of more than one physiological condition, and it makes possible the control of a graded series of responses by virtue of the phase angles between the daily rhythms of synergistic hormones. Temporal patterns would seem as worthy of consideration as dose levels with regard to hormonal control of metabolic processes.

Summary. Daily variations in fattening responses to prolactin may be phased or driven by injections of adrenal steroids in a fish, *Fundulus grandis*, a lizard, *Anolis carolinensis*, and a pigeon, *Columba livia*. Daily injections of prolactin about 24 hr after injections of adrenocortical hormones favor the accumulation of fat stores; whereas daily injections of prolactin 6 hr after the adrenal steroids cause losses in fat. An experiment on the fish indicated that the rhythm of fat responsiveness to prolactin is capable of self-sustaining circadian oscillations on continuous light following initial synchronization by exogenous hydrocortisone. An experiment on the pigeon indicated that a daily rhythm of cropsac sensitivity to prolactin may also be driven by daily injections of corticosterone, but the temporal pattern of the adrenal steroid and prolactin that favors cropsac proliferation is not in the same phase relations with those that favor accumulation of body fat, liver fat, and increases in intestinal weight. It is concluded that a temporal synergism of prolactin and the adrenal steroids is an important organizational unit in the vertebrate system.

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