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ABSTRACT

The relationship between photoperiod, plasma concentration of ionic calcium and the histology of the prolactin-secreting cells of the rostral pars distalis of the pituitary gland, the Corpuscles of Stannius and the Ultimobranchial gland were investigated. Neither the plasma concentration of ionic calcium nor histologically apparent prolactin cell activity could be correlated with photoperiod. Some evidence of a photoperiodic effect on both the Corpuscles of Stannius and the Ultimobranchial gland was obtained. The expected reciprocal relationship between the activity of these glands was not obvious at the histological level.

Quantitative and qualitative analysis at the light microscope level revealed, however, that the hormone prolactin-secreting eta cells of the rostral pars distalis and the hypocalcin-secreting cells of the Corpuscles of Stannius may be arranged in a lamellar pattern comprized of synchronous bands of cells in like-phase of a secretory cycle consisting of four stages - synthesis, storage, release and reorganization. Such synchronized cell cycles in these glands have not heretofore been described in literature.

It is suggested that the maintenance of at least 25% of the cells in any one phase of the cycle ensures a supply of the required hormone at all times.
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Principal Endocrine Organs of the Goldfish

- Corpuscles of Stannius
- Urophysis
- Gonad
- Interrenal
- Chromaffin tissue
- Hypophysis
- Spinal cord
- Ultimobranchial gland
- Thyroid
- Endocrine pancreas
- Cloaca
- Aorta
- Heart
- Liver
- Spinal cord
- Kidney
- Gall bladder
- Intestine
- Exocrine pancreas
Introduction

Calcium ions are involved in a wide variety of physiological and biochemical functions, ranging from control of cell membrane permeability and regulation of the activity of certain enzymes, through blood coagulation and bone growth to muscle contraction and the conduction of nerve impulses (Christensen, 1975). As calcium is involved in so many processes, the control of calcium metabolism is therefore a critically important process. In most tetrapod vertebrates, including mammals, regulation involves among other factors the formation and release of the hormones parathormone, from the parathyroid glands and calcitonin from the "C" cells of the thyroid glands. Of these, parathormone exerts a hypercalcemic activity, i.e. calcium elevating effect, while calcitonin is hypocalcemic, i.e. calcium reducing in action, respectively. Their effects have been shown to stem from influences upon calcium absorption by the gut, calcium loss and recovery from the urine and calcium mobilization from or deposition in bone. The principal features of calcium homostasis in mammals and presumably other tetrapods, is summarized in the "butterfly model" of Arnaud (1978), which is reproduced in Figure 1. Each wing of the butterfly represents a negative feedback loop involving one of the hormonal target organs, i.e. bone, intestine or kidney. The
INCREASING BLOOD CONC.  DECREASING BLOOD CONC.

Fig. 1. Butterfly model of physiological events involved in mammalian calcium homeostasis.
central point at which all the loops meet depicts the controlled variable, plasma concentration of ionic calcium. Depression or elevation of this prompts alterations in the secretion of parathyroid hormone (PTH) and calcitonin (CT) and initiates the restorative effects depicted by the loops.

The endocrine and other mechanisms involved in calcium regulation by Teleosts and other fishes are less well understood at present. Assuming that calcium is important in a wide range of processes, its control is likely to be as critical to piscine vertebrates as it is to tetrapods. A more complex problem, however, faces these aquatic organisms because, unlike terrestrial vertebrates, they are in direct contact with a medium whose calcium content may differ substantially from that of their body fluids. For example, in normal seawater (35%), calcium levels are approximately 0.4 g L\(^{-1}\) and are relatively constant and they exceed the values typically encountered in the blood of marine fishes by a factor of 3 to 4. In freshwater lakes and rivers, calcium levels are low by comparison with plasma concentrations and are more variable. Wetzel (1975) cites an average value for all North American waters of about 20 mg L\(^{-1}\). He also provides data for seasonal and vertical variations in a typical oligotrophic, hardwater lake (Lawrence Lake, Michigan) and a relatively soft-water eutrophic lake (Wintergreen Lake, Michigan). In the former, calcium varied from a surface value of about 40 to bottom (12 M) concentrations of up to 75 mg L\(^{-1}\) during
the winter months. Corresponding summer levels were approximately 60 to approximately 85 mg L\(^{-1}\). By contrast, in Wintergreen Lake, the summer calcium concentration ranged from 0 (surface) to 50 mg L\(^{-1}\) at the bottom (5 M). In winter this range was reduced to 30-50 mg L\(^{-1}\) with the maximum concentration occurring at an intermediate depth (3 M). Therefore, the freshwater fish is faced with the problem of regulating calcium under circumstances in which it tends to lose calcium and in which environmental calcium concentrations are more variable.

Figure 2 represents a possible model, fashioned on that of Arnaud, of calcium regulation in fishes. This model, like that depicted in Figure 1 for mammals envisages plasma calcium concentration as the outcome of a number of interacting physico-chemical and physiological-biochemical processes, integrated in some instances, by endocrine action. In fishes, as in mammals, bone serves as a calcium sink - accepting excess calcium when blood levels are above normal, releasing it into the plasma when calcium levels fall. In the case of the fishes, the scales and, perhaps to a lesser degree, mucous, may serve much the same function. To a large degree, but not exclusively, uptake and release of calcium from scales and, in particular, from bone can be regarded as passive processes. These are, however, amenable to endocrine modification.

Physiological transfers may take place at several points
Fig. 2. A Model of physiological events involved in piscine calcium homeostasis.
in the system - these are the gills, the gut tract and the kidneys. Because plasma calcium levels will ordinarily exceed those of the medium, a spontaneous efflux from, for example, the gills would be expected to and actually does occur. The extent to which the calcium loss takes place is, however, governed by a number of factors, two of which are gill calcium permeability and cardiovascular activity. A reduction in permeability leads to a drop in the rate of calcium loss even if the plasma:medium calcium concentration gradient remains constant. If there is an increase in cardiovascular activity or an elevation in temperature, there will be increased calcium depletion if the permeability of the respiratory surface and the concentration gradient driving efflux of calcium remain stable. Absorption from the gut tract depends in the first instance, upon the availability of calcium, i.e. upon the calcium content of food. Other determinants in the uptake process are the calcium permeability of the intestinal surface and the rate of blood flow to the gut tract. Many of these considerations apply to the kidney also. The rate at which calcium is passed into the primary urine depends on plasma concentration and glomerular filtration rate. Renally initiated changes in calcium economy are frequently encountered since glomerular filtration rate is intimately involved in (among other things) the regulation of moisture content. For example, in the carp, *Cyprinus carpio*, an
increase in environmental temperature of $10^\circ C$ results in a doubling of the urine flow in an attempt to relieve the water-loading induced by the increase in respiratory activity and there is corresponding increase in urinary calcium loss (Houston, 1973).

It becomes obvious that calcium transport against existing concentration gradients must take place, if overall calcium balance is to be maintained. This may occur at the gills and gut, and in relation to bone and possibly scales and skin as well (Simmons, 1971).

The role of the endocrines in calcium regulation can, therefore, involve a wide variety of target organs. The most obvious ones include activation or suppression of calcium transport at the several sites where this takes place and/or alterations in membrane permeability to calcium ions. Less direct effects can be considered as well. By stimulation or suppression of branchial, skin or gut mucous production, the effective permeabilities of these surfaces may be influenced. Alterations in cardiac function, blood pressure and/or blood flow distribution might significantly influence the rates of both calcium uptake and loss.

The problem is a complex one: there are many uncertainties regarding the actual mechanisms effected by hormonal elements of the calcium regulating system and compounding the latter are the uncertainties regarding the calcium
regulating endocrine organs in fishes and the relative importance of the roles which they play in this process. At present, three organs have been implicated. Among these are the eta or prolactin-secreting cells of the pituitary gland. These cells are known to exert a hypercalcemic or calcium-elevating effect. The Corpuscles of Stannius and the ultimobranchial gland, on the other hand, are believed to have a calcium-reducing or hypocalcemic action. Possible interactions of these organs, as suggested by earlier investigations, have been incorporated in Figure 2.

The principal goal of the present study was to assess the involvement of these endocrine organs in the regulation of calcium in the goldfish, Carassius auratus. In prior studies a variety of techniques have been used for this purpose. Many have involved what might be considered "invasive" techniques which included surgical removal of glands, injection of hormones and ectopic transplants of organs. It has been subsequently demonstrated (Houston, et al., 1971) that these types of procedures produce marked and persistent changes in water-electrolyte balance, including both the concentration and distribution of calcium ion. Therefore, it was decided to employ a "non-invasive" approach. Of the various factors which could be employed to alter endocrine function related to calcium balance, the most obvious is photoperiod. Rasquin and Rosenbloom (1954)
established that such effects possibly existed.

In the present study, it was hoped that through the manipulation of photoperiod, modifications in glandular activity would occur which would be reflected in plasma concentrations of ionic calcium. Experimental protocol involved the acclimation of a number of fish to several photoperiods, ranging from total darkness to continuous light, the determination of plasma calcium levels and an attempt to assess by histological techniques the activities of the pituitary, Corpuscles of Stannius and ultimobranchial gland in relation to calcium levels.

During this study, it became apparent that the cyto-architectural picture suggested by the observations made was not totally consistent with the classically held view of these organs. Therefore, it was decided to extend the study in this respect. In addition to exhaustive histological examination, emphasis was placed on the organization of prolactin cells within the pituitary gland, the epithelial cells lining the tubules of the Corpuscles of Stannius and the cellular arrangement within the ultimobranchial gland. This aspect of the research (for pituitary glands and the Corpuscles of Stannius) was facilitated by a Quantimet 720 Computer Image Analyzer, a system which can be utilized to provide semi-quantitative information on histological material.
Endocrine functions in fishes have been the subject of intensive studies in recent years and an extensive literature now exists. The magnitude of this can be appreciated by reference to a series of reviews prepared for inclusion in Vol. 2, Fish Physiology, edited by W.S. Hoar and D.J. Randall, a decade ago. At that time, Ball and Baker (1969) and Ball (1969), were able to cite 331 and 152 then current references in relation to the general anatomy and histology of the pituitary gland and prolactin functions respectively. Some 82 references were considered by Copp (1969) in reviewing the ultimobranchial gland and calcium metabolism in fishes, while Jones, et al. (1969) referred to 235 papers in their review of the Corpuscles of Stannius, adrenocorticotropin and the teleostean adrenocorticosteroids. More than one hundred references were cited by Fleming (1974) in an article devoted largely to calcium metabolism in fishes. Since the publication of these reviews, the volume of available information has expanded steadily. Therefore, no attempt has been made to exhaustively review this massive literature. Instead selected studies pertinent to the topic of this thesis have been summarized as background material. Four principal areas have been considered: (1) calcium distribution and alterations in calcium levels under various environmental conditions, (2)
general organization of the pituitary with emphasis upon the prolactin cell and its effects upon calcium metabolism, (3) the general organization and functions of the Corpuscles of Stannius in relation to calcium metabolism and (4) the general organization and functions of the ultimobranchial gland in this context. An attempt has been made to integrate earlier observations in relation to the tentative model of teleostean calcium regulation depicted in Figure 2 i.e. that based on the Armand 'butterfly' diagram previously considered.

I. **CALCIUM METABOLISM IN FISHES**

Fleming (1974) has recently reviewed several features of calcium metabolism in fishes. The largest proportion of the total amount of calcium in these animals is found in the skeleton. In the instance of the euryhaline killifish, *Fundulus kansae*, the skeleton accounts for 78.1% and skin and scales for 19.3% with the remainder (2.6%) allocated to blood and soft tissues. Similar values have been reported by Berg (1968, Mem. Inst. Ital. Hydrobiol., 23 : 161-196, reference not available, reviewed by Fleming, 1974) for the goldfish. In specimens ranging in weight from 9 to 14 g, bone contained 72% of total calcium, skin and scales 22% and the soft tissues and blood 6%.

It has been suggested that calcium sequestered in bone and scales is no longer available for exchange with soft tissues,
i.e. that calcium movement into these areas is essentially a one-way process and that such transfer of calcium as does occur involves only two basic shifts: environment-blood, blood-soft tissues. Both Fleming (1974) and Berg (1968; cited in Fleming, 1974) have examined this through the use of radio-calcium, following uptake and subsequent movement of this isotope from these areas. The estimated half-life (the time for exchange of 50% of the calcium present) of bone calcium ranged from approximately one year in the killifish to 246 days in the goldfish. Corresponding values for skin and scales were 180 days (killifish) and 115 days (goldfish). Accordingly, while movement is slow it nevertheless does occur. Much more rapid movement takes place in the soft tissues. In the killifish, for example, the mean half-life for all soft tissue calcium was estimated to be ~170 hours or about 7 days.

Calcium levels have been determined for several tissues in the rainbow trout, *Salmo gairdneri* (Murphy and Houston, 1977) and give some indication of the concentrations which can be expected in fishes. Epaxial muscle, which represents about 70% of body weight, is characterized by values ranging from 5.4 ± 0.30 to 7.0 ± 0.40 m Equiv kg⁻¹ under different experimental conditions. In a more limited study of the carp, *Cyprinus carpio* calcium levels in muscles ranged from 10.4 ± 1.0 to 14.7 ± 2.6 m Equiv kg⁻¹. In rainbow trout liver—approximately 4 to 6% of body weight — concentrations varied
from $0.6 \pm 0.07$ to $0.9 \pm 0.07$ m Equiv kg$^{-1}$. In heart muscle (trout) values of $2.2 \pm 0.66$ to $6.2 \pm 0.66$ m Equiv kg$^{-1}$ were observed. Much of this calcium is, however, bound to protein and other cytoplasmic constituents. Christensen (1975), for example, suggests that unbound and thus freely diffusible calcium may represent only $0.1$ to $1.0\%$ of the calcium associated with these tissues. Of the calcium present in the blood of rainbow trout and carp, about $20\%$ is associated with the red cells, with the remainder in the plasma (Houston and Smeda, 1979). Some of that in plasma is undoubtedly bound to plasma proteins. However, the proportion is probably small as plasma protein concentrations in fish are low by comparison with those of tetrapods (Feeney & Brown, 1974).

Thus, by far the largest amount of calcium in the fish is sequestered in bone, skin and scales, where it is only slowly accessible to the body fluids. Much of the remainder is within soft tissue cells or erythrocytes where the bulk is more-or-less tightly associated with proteins and other materials in the cytosol. That which circulates in the plasma and interstitial fluids is a relatively small proportion of the total, but is, nevertheless, critical for the plasma and interstitial fluids are the single avenue of exchange between all other regions in which calcium occurs.

An idea of the extent to which this accessible calcium can be depleted is gained by consideration of data for the
carp, a species which is closely related to the goldfish which was used in this study. Plasma calcium levels in this species range from 1.96 to 5.08 m Equiv L⁻¹, with a mean of about 4 m Equiv L⁻¹. The extracellular volume (plasma and interstitial fluid) in this species, as estimated from muscle, is roughly 5.7% of body weight (Houston et al., 1970; Houston & Smeda, 1979). It would therefore contain about 0.1 to 0.15 m Equiv L⁻¹ of calcium if, as it seems reasonable, the plasma and interstitial fluids are at roughly equal concentrations. A 500 g carp at 15°C produces about 25 ml of urine daily (1.8 ml kg⁻¹ hour⁻¹) at a concentration of about 1.5 m Equiv L⁻¹ (Houston, 1973). Urinary calcium loss therefore approximates 0.04 m Equiv L⁻¹ daily. Although these are approximate figures, they suggest that between one-quarter and one-third of the circulating calcium is excreted daily. Also, whatever is lost by diffusion from the gills must be considered. To stabilize calcium levels, an equivalent amount must be absorbed from water and nutrients, or mobilized from the soft tissue, scales or bone.

It is interesting to examine plasma calcium levels in relation to various environmental factors. Table I includes a number of values for marine and freshwater habitats. In Lophius piscatorius, L. americanus and Mureana helena, species which tend to occur in relatively low-salinity waters, plasma calcium levels are not greatly different from those occurring
in species which are found in high-salinity water, e.g. *Paralabrax clathratus*, *Sphaeroides maculatus*.

More variable values are seen in freshwater species. Shell (1959) recorded a seasonal minimum during August in the smallmouth bass, *Micropterus dolomieui*. Generally, the same was true for the carp (Houston, et al., 1970). Values obtained for summer populations of rainbow trout also tended to be lower than those of winter animals (Houston and Smeda, 1979) when equivalent temperatures were considered. There appear to be substantial grounds that a seasonal cycle exists. The two most obvious seasonal variants are temperature and photoperiod. In the case of the carp, increases in temperature were correlated with increases in the plasma calcium concentrations of summer and fall fishes (Houston, et al., 1970). Summer populations of this species also showed this trend to some extent (Houston & Smeda, 1979). Rainbow trout were more variable. Houston & Smeda (1979) showed no significant changes in either summer or winter fish. Murphy and Houston (1977), however, recorded modest but significant increases in concentration at higher temperatures. The latter study also involved manipulation of photoperiod in relation to temperature. Plasma calcium was consistently elevated in animals exposed to longer light periods (18L:6D versus 6L:18D) at specific temperatures. Also, specimens exposed to nominal 'winter' conditions (2°C, 6L:18D) exhibited lower calcium levels than did those held under a
'summer' temperature-photoperiod regime (18°C, 18L:6D). These studies suggest that calcium regulation can be influenced by season, by photoperiod and by temperature. However, none were designed to investigate hormonal influences upon calcium metabolism. Information related to endocrine effects is considered in subsequent sections dealing with specific endocrine organs.

**TABLE 1.**

Representative values for plasma calcium concentration in marine and freshwater species, millequivalents Ca\(^{2+}\) litre\(^{-1}\).

<table>
<thead>
<tr>
<th>Species</th>
<th>Plasma Ca(^{2+})</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Marine species</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lophius piscatorium</td>
<td>2.8</td>
<td>low-salinity species</td>
<td>Brull &amp; Nizet, 1953</td>
</tr>
<tr>
<td>L. americanus</td>
<td>2.2</td>
<td>&quot;</td>
<td>Forster &amp; Berglund, 1956</td>
</tr>
<tr>
<td>Mureana helena</td>
<td>3.87</td>
<td>&quot;</td>
<td>Robertson, 1954</td>
</tr>
<tr>
<td>Paralabrax clathratus</td>
<td>3.0</td>
<td>high-salinity species</td>
<td>Urist, 1962</td>
</tr>
<tr>
<td>Sphaeroides maculatus</td>
<td>3.4</td>
<td>&quot;</td>
<td>Eisler &amp; Edmunds, 1966</td>
</tr>
<tr>
<td><strong>Freshwater or</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Freshwater adapted</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coregonus clupeaformis</td>
<td>2.67</td>
<td>migratory-species</td>
<td>Robertson, 1954</td>
</tr>
<tr>
<td>Micropterus dolomicul</td>
<td>3.4</td>
<td>July</td>
<td>Shell, 1959</td>
</tr>
<tr>
<td>(smallmouth bass)</td>
<td></td>
<td>August</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>October</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>November</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>4.9</td>
<td></td>
<td>&quot;</td>
</tr>
</tbody>
</table>
TABLE 1 (continued).

Representative values for plasma calcium concentration in marine and freshwater species, millequivalents Ca^{2+} litre^{-1}.

<table>
<thead>
<tr>
<th>Species</th>
<th>Plasma Ca^{2+}</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Freshwater, or freshwater adapted species</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Megalops atlanticus (shad)</td>
<td>2.5</td>
<td></td>
<td>Urist, 1961</td>
</tr>
<tr>
<td><em>Salmo gairdneri</em> (rainbow trout)</td>
<td></td>
<td></td>
<td>Murphy &amp; Houston, 1977</td>
</tr>
<tr>
<td>&quot;</td>
<td>4.2</td>
<td>2° C, 18L:6D</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>3.4</td>
<td>2° C, 6L:18D</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>5.0</td>
<td>18° C, 18L:6D</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>4.8</td>
<td>18° C, 6L:18D</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>4.65</td>
<td>20° C, summer</td>
<td>Houston &amp; Smeda, 1979</td>
</tr>
<tr>
<td>&quot;</td>
<td>4.65</td>
<td>10° C, summer</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>4.67</td>
<td>18° C, summer</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>5.02</td>
<td>2° C, winter</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>5.10</td>
<td>10° C, winter</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>4.87</td>
<td>18° C, winter</td>
<td>&quot;</td>
</tr>
<tr>
<td><em>Cyprinus carpio</em> (common carp)</td>
<td>1.96</td>
<td>2°, summer</td>
<td>Houston, <em>et al.</em>, 1970</td>
</tr>
<tr>
<td>&quot;</td>
<td>2.42</td>
<td>7°, summer</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>3.88</td>
<td>4°, fall</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>4.29</td>
<td>17°, fall</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>4.88</td>
<td>27°, fall</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>5.08</td>
<td>33°, fall</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>3.82</td>
<td>27°, winter</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>3.70</td>
<td>2°, summer</td>
<td>Houston &amp; Smeda, 1979</td>
</tr>
<tr>
<td>&quot;</td>
<td>4.30</td>
<td>16°, summer</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>4.22</td>
<td>30°, summer</td>
<td>&quot;</td>
</tr>
</tbody>
</table>
TABLE 1. (continued)

Representative values for plasma calcium concentration in marine and freshwater species, millequivalents Ca\(^{2+}\) litre\(^{-1}\).

<table>
<thead>
<tr>
<th>Species</th>
<th>Plasma Ca(^{2+})</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshwater, or freshwater adapted species</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tinca tinca (tench)</td>
<td>4.45</td>
<td>6(^{\circ}), summer</td>
<td>Meincke, 1970</td>
</tr>
<tr>
<td>&quot;</td>
<td>4.90</td>
<td>13.5(^{\circ}), summer</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>3.92</td>
<td>16(^{\circ}), summer</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

II. THE PITUITARY GLAND

General Morphology and Histology

In all vertebrates the pituitary develops from ectodermal tissue and includes two structurally-distinct regions, the adenohypophysis and the neurohypophysis (Turner & Bagnara, 1971). The former arises from buccal epithelium (somatic ectoderm) while the neurohypophysis develops from the ectodermal neural tube. Since this close association of essentially epithelial and neural elements persists throughout life, it is not surprising that epithelial cell activities, including secretion of hormones, are frequently intimately related to, and to a great extent controlled by nervous activities.

In the teleost fishes, the pituitary gland is normally suspended from the ventral aspect of the diencephalon by means of an infundibular stalk (Schreibman, Leatherland and McKeown,
Although overall morphology and internal cellular organization often differ from species to species the gland can ordinarily be differentiated on histological grounds into three principal regions. The adenohypophysis includes the more anterior pars distalis, made up of rostral and proximal or caudal components, and a more posteriorly positioned pars intermedia. Among cyprinid fishes, the group which includes the goldfish, the neurohypophysis forms an elongated axis around which the adenohypophyseal components are arranged (Holmes & Ball, 1974). The pars intermedia lies ventrad and adjacent to the proximal elements of the pars distalis, with the rostral component forming an "... incomplete ring embracing the neurohypophysis anteriorly..." (Olivereau, 1968).

The first detailed description of pituitary morphology in the goldfish appears to have been that of Bell (1938), who said that it was an irregular, solid, somewhat rounded mass measuring about 1.3 mm in length. He further described it as being located below the anterior brain case in a cavity, the myodom, which extended from the optic foramina to the medullary region. The infundibular stalk emerged from the brain case via a foramen in the parasphenoid bone and extended posteriorly as the pars nervosa. As mentioned earlier, the remaining elements are arranged around the axis provided by the neurohypophysis. The anterior elements of the gland are closely opposed to the ventral surface of the braincase and
close to the optic-pituitary foramen. This description was subsequently confirmed by Levenstein (1939) and Chavin (1956). Both authors also differentiated three principal cell types in the gland on the basis of their stain affinities, describing acidophilic, basophilic and chromophobic cells.

Little attention was given to the goldfish pituitary morphology for some years following these initial studies. Recently, however, Leatherland and his coworkers have undertaken comprehensive investigations involving both light and electron microscopy (e.g. Leatherland, 1972). In gross morphology, Leatherland describes the goldfish pituitary gland as an "...ovoid structure connected to the hypothalamus by an infundibular stalk and enveloped in a sella turcica ...". Using essentially cytological criteria, he confirmed the subdivision of the gland into four main regions on the basis of cell distribution patterns: Three adenohypophyseal components, the rostral pars distalis (RPD), the proximal pars distalis (PPD) and the pars intermedia (PI) and neurohypophysis (NH). A more detailed account of these areas follows.

Pars Distalis

Rostral Pars Distalis. The eta cells of the rostral pars distalis are regarded as the site of synthesis of the hormone prolactin. Histologically these cells are usually seen as semi-compact masses organized into cords and clumps of cells, or in a follicular arrangement. The former is considered to
be a distinguishing feature of more advanced teleosts, while
the follicular type of cell grouping is more typical of a
primitive state (Ball & Baker, 1969; Holmes & Ball,
1974).

The prolactin cells of goldfish are acidophilic or
erythrosinophilic in nature. In this they resemble the STH
(somatotrophic hormone) cells found adjacent to this area.
In terms of overall morphology, prolactin cells are sometimes
quite variable, ranging from oval to cuboidal or pyramidal in
shape. Sometimes they are near-spherical in form, with
diameters in the 5-8 μm range. Their nuclei are often round
(4-6 μm in diameter), but are also oval or kidney-shaped. The
cytoplasm of the prolactin cell is characteristically granular.
Evidence gained from electron micrographs (Leatherland, 1972),
indicates that these granules are spherical, electron-dense
structures ranging in diameter from 2100-3500A and either
bound to, or closely associated with membranes. Fluorescent
ovine prolactin antibody associates specifically with these
granules providing strong evidence that the hormone is located
here (Ball & Baker, 1969; Emmart, 1969; Mckeown & Van
Overbeeke, 1971). The studies of Emmart (1969) are interesting
as they indicate that, while all of the cells in the goldfish
rostral pars distalis appear to contain prolactin, the
acidophils immediately adjacent to a 'transitional zone'
between the rostral and proximal areas of the adenohypophysis
provide a more intense reaction.

Interspersed among the prolactin cells are chromophobic cells. These occur both singly and in follicular formations around the blood vessels which ramify throughout this area. In addition, they are found in association with cords of prolactin cells and occasional basophilic cells in the proximal-rostral 'transitional zone' previously referred to. These cells, like the eta acidophils, have rounded nuclei and are characterized by numerous spherical, electron-dense and membrane-bound granules, Golgi bodies and mitochondria. Endoplasmic reticulum is plentiful as are large amorphous droplets. An ACTH and/or TSH-secreting function has been attributed to these chromophobes.

Electron microscope studies have revealed smaller numbers of three other cell types (Leatherland, 1972). One type commonly seen in specimens adapted to seawater, but not as numerous in freshwater-adapted fish, can be distinguished from prolactin and chromophobe cells on the basis of several characteristics: (1) smaller amorphous droplets and granules, (2) fewer mitochondria and (3) less abundant endoplasmic reticulum. Small angular cells are seen around the periphery of the rostral pars distalis and interspersed with the eta cells. These possess little cytoplasm and are essentially agranular. Finally, a fifth, basophilic cell is encountered in the midline and ventral portions of this region. Again,
this cell type is small and angular and possesses relatively little cytoplasm. The cytoplasm does, however, include granules and is similar to a basophil cell type found in the proximal pars distalis.

**Hormonal Release**

The release of adenohypophyseal secretory products by the goldfish may take place by at least two processes. In some instances and most commonly in the case of rostral eta cells, for example, Leatherland (1972) described granules enclosed in membranes lying between neurosecretory fibres and adjacent to blood cells. He therefore postulated that these were first extruded and then 'pinched off', as it were, outside the cell. Subsequent breakdown of the enclosing membrane and solution of granular product would be required to place the latter in the circulation, but the mechanism(s) by means of which this might take place are obscure.

An alternative and more common mode of release was observed in rostral acidophilic cells and also in rostral chromophobes, proximal pars distalis acidophilic cells and both of the cell types found in the pars intermedia. In this case, the granules first appeared to lose accompanying membranous elements. They then fused with the plasmalemma and the contents of the granules apparently passed through the latter - presumably by diffusion. In many instances, release
by this method was associated with structural modifications. It was noted earlier that the neuro- and adenohypophyses are intimately related by way of extensive interdigitations. At such points, complex double basement membrane systems are frequently seen, e.g., in the polyteriform fishes, *Calamoichthys calabricus* and *Polypterus palmas*, the bowfin, *Amia calva*, threespine stickleback *Gasterosteus aculeatus*, surfperch, *Cymatogaster aggregata* and kokanee salmon, *Oncorhynchus nerka* and in a number of tetrapod vertebrates as well (Lagios, 1968, 1970; Nagahama & Yamamoto, 1969; Leatherland, 1970, 1972). These adenohypophyseal-neurohypophyseal junctions include the basement membranes of adjacent epithelial and neural cells plus variable amounts of homogenous matrix without obvious internal structure. The thickness of the complex varied from $\sim 1000$ to $\sim 2000$ Å being greater and more electron dense in regions where the epithelial cells appeared to be in a state of active secretion. Leatherland (1972) has therefore concluded that at least some adenohypophyseal hormones are released into these junctional matrices and from there pass into the circulation. As was noted earlier, neural elements, and notably those of the A2 type, tend to synapse on the basement membranes of blood vessels and consequently may also release product in close opposition to the circulation.

Finally, there has been little or no information found
regarding the mechanism of granule release employed by the various basophil cell types.

**Pituitary Role(s) in Ionic and Osmotic Regulation**

In fishes, as in more highly evolved vertebrates, the hormones elaborated by the adeno- and neurohypophyses act indirectly or directly upon a wide range of biochemical and physiological activities. These have been extensively reviewed (Ball & Baker, 1969; Ball, 1969; Bentley, 1971; Holmes & Ball, 1974). Consequently, in this and the succeeding section, brief consideration is given first to the role of the pituitary in general osmo- and ionoregulatory processes, and second to the more specific question of pituitary influence upon calcium metabolism and regulation.

Much of the evidence pointing to involvement of the pituitary and specifically, the prolactin or eta cell components in water-electrolyte balance arises from three principal approaches, which have been employed both singly and in a variety of combinations. These include:

1. hypophysectomy of normally freshwater, marine and/or euryhaline fishes and exposure to various osmotic stresses. In such cases survival and variations in blood and (sometimes) tissue water and ion levels have been followed as have ionic fluxes.

2. hypophysectomy followed by replacement therapy under the circumstances previously noted.
(3) assessment of changes in glandular structure and apparent activity following osmotic transfer.

A number of euryhaline species - mainly but not exclusively cyprinids - appear unable to tolerate freshwater conditions following hypophysectomy. This is, for example, true of Fundulus heteroclitus, a species characterized by marked reductions in plasma osmolarity and plasma chloride and sodium levels, moderate increases in water content and little change in plasma potassium after this type of surgical intervention (Burden, 1956; Pickford & Phillips, 1959; Pickford, et al., 1965). Ultimately, death occurs in the absence of hormone replacement therapy. However, only prolactin alleviates these changes and does so in a manner which is prolactin dose-dependent. A variety of other hormones including adrenocorticotropic hormone, thyroid-stimulating hormone, thyroxine, growth hormone, Corpuscles of Stannius extracts and several others either have no effect, or at best, only minor effects on comparison with prolactin. Ectopic transplantation of pituitaries into hypophysectomized freshwater-adapted Poecilia formosa will also promote survival (Olivereau and Ball, 1966). Only the prolactin cells of such transplants exhibited signs of increased activity.

As initially noted, hypophysectomy does not necessarily have lethal effects on freshwater fishes. The freshwater-adapted stage of the migratory eel, Anguilla anguilla, is capable of prolonged survival following hypophysectomy and
this is true of the goldfish as well (Chavin, 1956; Olivereau & Chartier-Baraduc, 1966). Both, however, exhibit alterations in water-electrolyte balance not unlike those seen in animals unable to tolerate freshwater conditions following hypophysectomy, i.e. reductions in plasma sodium and chloride levels together with alterations in water balance which can be alleviated by ovine prolactin administration. Treatment of rainbow trout with ergocryptine (an ergot alkaloid which inhibits prolactin) also causes reductions in plasma and tissue ion levels (Brewer & Mckeown, 1978). Thus, the difference seems to lie in the sensitivity of the species rather than the effects of prolactin.

Transfers between media of varying salinity produce patterns of cytological variation which also suggest that prolactin is primarily involved in freshwater osmotic and ionic regulation. Thus, specimens held in freshwater as compared to brackish or full-strength sea water exhibit changes in cellular structure suggestive of increased cellular activity and these are largely confined to the prolactin cells (Ball & Olivereau, 1964; Olivereau & Ball, 1964; Ball, 1969). Transfer of Xiphophorus maculatus from fresh to sea water prompts regressive changes in prolactin cells which are reversed upon return to seawater (Holtzman & Schreibman, 1975). Much the same is true of a variety of other more-or-less euryhaline species including Fundulus kansae, Mugil cephalus and Tilapia
mossambica (Olivereau & Ball, 1964; Abraham, et al., 1967; Olivereau, 1968b; Ball & Fleming, 1968; Dharmamba & Nishiaka, 1967). In species which are periodically euryhaline (e.g. migratory salmon, eels), the freshwater-adapted stages usually have more, and apparently more active prolactin cells than do those in seawater (Olivereau, 1954, 1966; Ball & Olivereau, 1964; Van Overbeeke & McBride, 1967). Tilapia species (T. zillii, T. nigra, T. leucosticta, T. alcalica, T. grahami) inhabiting 'normal' as opposed to 'soda' lakes (those high in sodium carbonate content) have been examined by Leatherland, et al., (1974). Those which are normally found in freshwater exhibited the usual pattern of eta cell morphology; 'soda' lake species were more similar in many respects to marine forms. Transfer of the latter to less saline conditions was accompanied by increase in the number of eta cells present as well as degranulation and other indications of hyperactivity. Briefly stated, the 'soda' inhabitants tended to revert to a more typically freshwater state.

There seems to be little question as to the importance of the pituitary and specifically the hormone prolactin in the maintenance of water-electrolyte economy in stenohaline freshwater animals and in freshwater-adapted euryhaline and migratory species.

The site or sites of prolactin action have not as yet been entirely clarified, nor have the mechanisms by means of
which prolactin acts. Nevertheless, it is apparent that one target organ is the gill system. The effects of prolactin at this site may, however, differ in different species. Maetz, et al., (1967) and Ensor & Ball (1972) found that hypophysectomized Fundulus heteroclitus and Poecilia latipinna exhibited massive increases in branchial sodium efflux, which could be corrected by prolactin administration. This would suggest an influence upon the sodium permeability of the gills. By contrast, in Fundulus kansae hypophysectomy leads to an increase in urinary sodium loss and a reduction in branchial sodium uptake, but little change in passive efflux. Prolactin administration, in this instance, appears to increase renal recovery and the branchial uptake of this ion. It is not presently certain whether these effects are mutually exclusive, nor have other possible actions been excluded. Those which have been identified are obviously consistent with the maintenance of electrolyte levels under circumstances prompting salt depletion.

Finally, some comments should be made with respect to photoperiodic influences upon prolactin involvement in the iono-(osmo-) regulatory process. Evidence of this was observed by Lam & Hoar (1967) as a consequence of investigations upon seasonal differences in the ability of the stickleback, Gasterosteus aculeatus, to adapt to freshwater. Winter animals transferred to freshwater exhibit sharp decreases in plasma
osmolarity and plasma sodium and chloride levels and frequently die. Spring and summer fish, however, are usually able to adapt to freshwater successfully. Administration of prolactin to winter animals prior to transfer reduces osmoregulatory dysfunction and improves survival (Lam & Leatherland, 1969). Lam and Hoar (1967) postulate that the intact winter fish is, in effect, "physiologically hypophysectomized", and suggest that reduced photoperiod may contribute to this. Subsequently, Lam (1972) demonstrated that maintenance of these animals during the fall under relatively long photoperiod improved their osmoregulatory capabilities and that this effect could also be achieved in short photoperiod specimens through prolactin treatment. Confirmation of this was subsequently made by Mckeown & Peter (1976) who utilized radioimmuno-assay methods to assess photoperiod-temperature effects upon serum and pituitary prolactin levels. Increases in photoperiod were accompanied by increases in prolactin concentration and diurnal cycles in serum prolactin content could be linked to light-dark transitions as well. Thus, there can be little doubt as to photoperiodic influences upon prolactin.

Pituitary and Calcium Regulation

Although fishes lack the parathyroid system used by tetrapods in hypercalcemia regulation, it seems highly unlikely that any vertebrate organism living in a calcium-depleting
environment would lack a means of elevating serum calcium levels. There is, in fact, substantial evidence which indicates that teleost fishes possess a vitamin D3 system which is at least generally similar to that of mammals, i.e. it increases calcium absorption from the gut and the mobilization of calcium from bone. It is apparent from the mammalian literature (Lorenc, 1977) that vitamin D3 is hepatically converted to 25-hydroxyvitamin D3 and that this is subsequently converted to 1, 25-dihydroxyvitamin D3 by the renal enzyme 25-hydroxyvitamin D3-1-hydrolase. It is 1, 25-dihydroxyvitamin D3 which is active in the absorptive and mobilization processes previously suggested.

Studies by Henry and Norman (1975) and Kenny, et al., (1977) have confirmed the presence of this key enzyme in the kidney of the goldfish and also in a variety of other freshwater and marine species (carp, Cyprinus carpio; rainbow trout, Salmo gairdneri rockfish, Sebastus carnatus; sea bass, Epinephelus labriformis; sculpin, Scorpaena guttata; bonito, Sarda chiliensis; kelp bass, Paralabrax clathratus; killifish, Fundulus heteroclitus). Furthermore administration of 1, 25-dihydroxyvitamin D3 to eels prompts the expected response (McIntyre, et al., 1976). Therefore, there can be little doubt as to the ability of fishes to produce the active form of vitamin D3 and there is at least some evidence to suggest that it is linked to calcium metabolism.
An hypophyseal and specifically prolactin involvement in hypercalcemic regulation in fishes presently seems probable. Pang and his coworkers (Pang, et al., 1971; Pang, et al., 1973) have demonstrated hypocalcemia and the occurrence of hypocalcemic tetanic seizures in hypophysectomized killifish maintained in calcium-free seawater. Administration of killifish pituitary extracts alleviated these symptoms. Hypophysectomy and replacement therapy confirmed a hypercalcemic pituitary role in soft freshwater-adapted Fundulus diaphanus and also gave some indication that pituitary involvement in calcium regulation becomes more important as environmental calcium levels are reduced (Pang, et al., 1973). Replacement therapy studies provided evidence that it is prolactin which is the pituitary principle involved in calcium regulation (Pang, et al., 1973). Interestingly enough, similar studies (hypophysectomy/prolactin administration) indicated a comparable involvement of prolactin in calcium metabolism in urodele amphibians (e.g. Cryptobranchus alleganiensis, Necturus maculosus, neotenic Ambystoma tigrinum), species lacking parathyroid glands and also in the bullfrog, Rana catesbianna which possesses parathyroid glands (Baksi; et al., 1978; Pang, 1978, personal communication to A.H. Houston).

In addition, prolactin was recently shown to stimulate renal formation of active vitamin D3 in birds (Spanos, et al., 1976 a, b; Baksi, et al., 1978). A similar demonstration does
not appear to have been made in fishes as yet. These studies on birds do raise the possibility that the hypercalcemic influence of prolactin may be mediated, in part at least, through some influence upon renal formation and/or release of 1-25-dihydroxyvitamin D₃.

The foregoing investigations point strongly to the probability of a specific and significant prolactin involvement in the hypercalcemic aspects of serum calcium regulation and it may be speculated that the pituitary-prolactin system constitutes the critical hypercalcemic regulator in fishes and in parathyroprivic (i.e., urodele) amphibians, thus reflecting a primitive regulatory arrangement which retains a minor role in more advanced tetrapods, a role which may possibly be mediated by way of the vitamin D₃ absorption-mobilization system.


V. **Corpuscles of Stannius**

**General Morphology and Histology**

The Corpuscles of Stannius (CS) were named after their discoverer, Stannius 1839; cited in Tomasulo, et al., 1970), who described the occurrence of these small bodies within a variety of fishes. Huot (1898; cited in Tomasulo, et al., 1970) demonstrated that embryologically these bodies arise from the pronephric ducts. Alternatively, Krishnamurthy (1967) discovered that these bodies can also develop from the mesonephritic tubules. Corpuscles of Stannius are found only in Holostei and Teleostei. Homology with any vertebrate gland is not yet established (Krishnamurthy, 1967). Their function as endocrine organs is poorly understood. Ultrastructural studies suggest that they are protein-secreting endocrine glands (Oguri, 1966). Recently, investigators have shown that the Corpuscles of Stannius secrete a factor which is responsible for electrolyte homeostasis with particular emphasis on the regulation of calcium (Pang, 1973; Fenwick, 1974).

Corpuscle number varies among species of fishes and frequently among individuals of the same species. For example in *Salmo gairdneri* this number varies from four to six; in
Onchorhynchus tshawytscha from four to five (Krishnamurthy & Bern, 1969); in C. auratus there are two corpuscles and, infrequently, three. Generally, the Corpuscles of Stannius are surrounded by a connective tissue capsule. Septa which arise from this capsule penetrate the cellular mass and thereby divide it into lobes which consist of cords or lobules. Krishnamurthy & Bern (1969) recognize four structural patterns within the CS of fishes. Definition as to Type is broadly based on the regular, irregular or incomplete appearance of the lobules. The structural pattern of the Corpuscles of Stannius of the goldfish is classified as Type I. Histologically, the Corpuscles of Stannius are composed of compact cords lined by a single layer of cells along a connective tissue septum. This arrangement results in an overall tubular appearance of the gland. The cells are arranged in radial fashion, one cell deep, around the circumference of the tubules. The vascular poles of the polygonal-shaped cells rest on a basement membrane which in turn abuts on a capillary. The apices of these cells point into the central region of the pseudotubules. Cells may be 6 -10 μm in diameter, with slightly eccentric, round to oval nuclei which are approximately 4 - 6 μm in diameter. Krishnamurthy & Bern (1971) suggest that innervation is of critical importance in the regulation of endocrine gland function. Neurons may be located in the connective tissue
capsule as well as among the gland cells. Nerve fibers can penetrate the capsule and enter the corpuscles along with the blood vessels. Possible termination of processes on the cells may also occur. Silver-staining, fluorescence and electron-microscopic approaches for further elucidation of the precise nature of the nerve supply are, however, necessary.

Electron microscope observations on the Corpuscles of Stannius in the goldfish (Oguri, 1966) suggest that the secretory granules contained within the cytoplasm of these cells are 300 - 1000 μm in diameter; most are round in shape. Each electron-dense granule is enveloped in a limiting membrane. The interior of the granule is homogeneous and osmiophilic. Oguri (1966) detected the same type of granule in the cells from the striped mullet, Mugil cephalus, Japanese eel, Anguilla japonica, and catfish, Parasilurus asotus, as did other researchers during their investigations into the fine structure of the cells of the Corpuscles of Stannius in the guppy, Lebistes reticulatus, (Tomasulo, et al., 1970), Atlantic salmon, Salmo salar L., (Carpenter & Heyl, 1974), killifish, Fundulus heteroclitus, (Cohen et al., 1975), and the toadfish, Opsanus tau, (Bhattacharyya & Butler, 1978). Oguri (1966) suggests that in C. auratus two types of cells can be distinguished. Although both cell types contain mitochondria, a Golgi apparatus and numerous
ribosomes in the cytoplasm, one cell type possesses secretory granules and a poorly developed endoplasmic reticulum ($S_1$). The other type of cell ($S_2$) exhibits a well-developed rough endoplasmic reticulum, but contains few or no secretory granules. He suggests the presence of an intermediate cell ($S_m$) as well and postulates that the variability in cellular appearances is possibly due to differences in the functional state of the same cell.
Corpuscles of Stannius and Calcium Metabolism

Surgical removal of the Corpuscles of Stannius (stanniectomy) can prompt changes in a variety of electrolytes (Fontaine, 1964, 1967; Butler, 1969; Chan, 1970, 1972; Chan, et al., 1967). These effects are, however, noteworthy for the inconsistency of their occurrence and magnitude. It may be that such changes reflect traumatic response to the procedures employed (e.g. Houston, et al., 1971) rather than the influence of the gland itself. On the other hand, ablation of the corpuscles has been consistently correlated with hypercalcemia in virtually all species thus far investigated; Anguilla anguilla, A. japonica, A. rostrata, C. auratus, C. carpio, F. heteroclitus, Cicyases sanguineus (Fontaine, 1964, 1967; Chester-Jones & Henderson, 1965; Chester-Jones, et al., 1967; Chan & Chester-Jones, 1968; Chan, et al., 1967, 1969; Ogawa, 1968; Butler, 1969; Chan, 1970, 1972; Lopez, 1970; Pang, 1971a; Fenwick & Forster, 1972; Fenwick, 1974; Galli-Gallardo, et al., 1977).

Interestingly, the hypercalcemic response is notably higher in stanniectomized animals held in high-calcium medium.

Three sites of action have been considered in relation to the Corpuscles of Stannius. Lopez (1970, 1973), has demonstrated alterations in the bone of an anguillid species (A. anguilla) following stanniectomy. Renal functions have also been examined in this respect, although the results have
not been consistent. For example, Chan and his coworkers (Chan, 1972; Chan, et al., 1969) reported a decline in renal calcium output following stanniectomy. Butler (1969) and Fenwick (1974) on the other hand, found no effect on actual increases in calcium loss rates. Branchial effects have also been investigated. In the stanniectomized, but otherwise intact eel, Fontaine, et al., (1972) noted an increase in calcium uptake. More recently, using an isolated-perfused eel gill preparation, Fenwick & So (1974 a, b; So & Fenwick, 1977) were able to demonstrate that stanniectomy prompted an increase in calcium uptake from the environment and also stimulated CA$^{2+}$-activated ATPase activity in the gills. Thus, there is evidence that the hypercalcemia of the stanniectomized fish may stem from decreased renal excretion, increased branchial absorption and/or mobilization from bone.

There can be little doubt as to the hypocalcemic effects of the Corpuscles of Stannius. Work by Pang, et al., 1974, suggested that the active substance (which they term 'hypocalcin') was a low molecular weight ($\sim 1000$) protein, stable in dilute urea but relatively heat labile. More recently, the work of Copp and his group has clarified much of the molecular structure of Corpuscle of Stannius active principle (Fenwick, pers. comm.). The Corpuscles of Stannius are also known to produce a renin-like material which has a
pressor (blood-pressure altering) action (Chester-Jones, et al., 1966; Sokabe, et al., 1970; Nakajima, et al., 1971). The relationship between the hypocalcemic and pressor effects remains unclear at present. Studies by Pang, et al., (1979) and Okagi, et al., (1977; cited in Pang, et al., 1979) can be interpreted as suggesting that the Corpuscles of Stannius produce and release a protein which then converts a plasma protein to a substance with pressor and, in bony fish, hypocalcemic actions. These questions, however, remain unresolved at present.
IV. The Ultimobranchial Gland

General Morphology and Histology

Van Bemmelen (1886; cited in Ball & Baker, 1969) first described these glands in elasmobranchs and chimaeroids. Due to their location on the pericardium, he referred to them as suprapericardial bodies. Griel (1905; cited in Pang, 1971b) introduced the term ultimobranchial body which is presently used. This name is perhaps more appropriate since it not only describes the embryonic origin of the gland, but also its anatomical position in most vertebrates. Watzka (1933; cited in Ball & Baker, 1969) searched for this gland in representatives from all vertebrate classes. Although he was unable to demonstrate its presence in cyclostomes - hagfish and lampreys - it was present in all jawed vertebrates.

Originally, the ultimobranchial developed as a follicular gland. This arrangement persists in elasmobranchs, amphibians and reptiles. Occasionally follicles are seen in avian ultimobranchial glands. In man, the ultimobranchial merges with the thyroid (Copp, 1969).

In most teleost fishes, the UBG (ultimobranchial gland) normally exists as a sheet of tissue which is located in the interseptum between the pericardial and peritoneal cavities, and lies in a position ventral to the esophagus. The UBG
may be single or paired. Frequently, singly-occurring glands are subdivided into left and right portions by a connective tissue septum. In trout (Robertson, 1967, 1969; cited in Pang, 1971b) and eel (Lopez, et al., 1968) the UBG has a follicular structure. In the killifish, Fundulus heteroclitus, it appears as cell cords (Pang, 1971b). Oguri (1973) describes the histological appearance of this gland in the goldfish as variable in appearance - showing lobular, follicular and cord-like arrangements. Further research into the problem has suggested that, in teleosts, the UBG is normally composed of follicles or cell cords. These cords of cells can, however, be transformed into follicles under the appropriate stimulatory conditions which induce a hypertrophization of the gland (Pang, 1971b).

Oguri (1973) investigated seasonal histological changes in the UBG of the goldfish. As stated previously, the ultimobranchial cells occur in lobular, follicular or cord-like arrangements. Nerve cells are present in or around the UBG parenchyma. Extensive vascularization by blood capillaries punctuates the cord-like arrangement of the gland. Oguri (1973) describes the cell cytoplasm as either basophilic or acidophilic, depending on the fixative employed. He was unable to distinguish secretory granules with his staining methods. Nuclei are vesicular, round or oval in shape and occupy the distal portion of the cell in the lobular and
follicular arrangements of the gland, whereas in the cord-like arrangement, nuclei are round to oval and are more centrally located. In August, October and December, the cells of the goldfish UBG are arranged in cord-like array. In February and March, a hyperplasia is evident and the gland becomes more lobular. In May, which corresponds to spawning season, a definite follicular arrangement of the parenchyma is obvious. Although Oguri (1973) does not describe the cells comprizing these glands in any detail, examination of the light micrographs in this paper reveals that those possessing an epithelial-like appearance comprize what is termed the cord-like arrangement of the gland. Cuboidal and/or columnar cells compose those glands termed hyperplastic (lobular). Finally, those glands described as follicular exhibit two different portions of glandular tissue - an epithelium of low to high columnar cells and acinar-like structures which are composed of cuboidal basal cells and plump, clear central cells.

Yamane and Yamada (1977) examined the histological changes in the UBG through the life history of the Masu salmon Oncorhynchus masou. They found that, in parrs, maturation of the UBG parenchymal cells progresses with the growth of the fish. Gland cells increase in height during the smolt stage. In freshwater ascending fish, gland cell nuclei elongate and cytoplasm is of basophilic cast and abundant blood capillaries become apparent. Examination of electron
micrographs reveals a well-developed, granular endoplasmic reticulum. This suggests that protein synthesis may be taking place. The height of the gland cell reaches its maximum in the pre-ovulatory female salmon and is subsequently reduced post-ovulation. These same features do not occur in the male glands. Deville & Lopez (1970) studied changes in the UBG during the life cycle of Salmo salar L. Their results are in agreement with the previous studies cited which suggest that extensive changes in the histological appearance of the UBG occur in response to an appropriate stimulus or challenge.
The Ultimobranchial Gland and Calcium Metabolism

The ultimobranchial gland has been shown to produce calcitonin (Copp, et al., 1967; Pang, 1973; McMillan, et al., 1976; Tisserand-Jochem, et al., 1977) which is an accepted hypocalcemic factor in mammals (Kalu, et al., 1976; Barlett & Theriez, 1976; Cutler, et al., 1977) and in sub-mammalian vertebrates (Belanger, et al., 1972; Sasayama & Oguro, 1976). The gland is thought to be homologous to mammalian parafollicular "C" cells (Pearse & Carvelhiera, 1967).

Experimental attempts to verify a hypocalcemic response analogous to that in mammals with calcitonin have yielded confusing and even contradictory results. Studies on catfish, killifish, salmon and trout indicated that mammalian and salmon calcitonin had no hypocalcemic effect (Pang & Pickford, 1967; Pang, 1971b; Lopez, et al., 1971) whereas Louw, et al., (1967) and Chan (1969) elicited significant reductions in the catfish, Ictalurus melas, and the eel Anguilla anguilla respectively. A hypocalcemic response was also reported in eels treated with calcitonin (Pang, 1971b; Lopez, et al., 1976). Orimo, et al., (1971) were unable, however, to demonstrate a hypocalcemic effect using purified Japanese eel calcitonin on A. japonica. Pang's (1971b) and Chan's (1972) research supported this finding. Fleming, et al., (1973) suggested that plasma calcium levels may not be a sensitive enough indicator of the effect of calcitonin in fishes.
Partial ultimobranchiallectomy produced a significant increase in plasma calcium and chloride levels in goldfish which were transferred from fresh to one third salt water. However, no response was elicited in animals maintained in freshwater (Fenwick, 1975). In the goldfish, hypercalcemia induced by excision of the Corpuscles of Stannius, elicited none of the histological changes that might have been expected as responses by the ultimobranchials to the challenge of high blood calcium levels if, in fact, the gland was secreting a hypocalcemic factor (Yamane & Yamada, 1977).

Despite these contradictions, one of the roles of the ultimobranchial is still thought to be the production of a hypocalcemic hormone. Recent histological work has provided support for this hypothesis. Peignoux-Deville, et al., (1975) were able to correlate cell types in various stages of secretion and overall epithelial appearance with experimental conditions. Injections of salmon calcitonin induced atrophication of the silver eel's ultimobranchial epithelium relative to that of control animals and carp pituitary extract injection, which induced hypercalcemia, resulted in a hyperplasia of the epithelium. Examination of salmon ultimobranchial glands during migration from salt to freshwater (Yamane & Yamada, 1977) and from fresh-to-salt-to-freshwater (Deville & Lopez, 1970) has correlated histological changes indicative of responses of the gland with environmental
challenge. As well, in some instances hyperplasia related probably to yolk deposition in female fish has been observed. Evidence exists that calcitonin may have yet other functions in fish. Deftos, et al., (1974) and Orimo, et al., (1977) measured circulating levels of calcitonin and suggested that this hormone may be related to sexual maturation. Many as yet unidentified functions of calcitonin possibly await discovery and further studies are necessary before the hypocalcemic effects of calcitonin can be properly designated to piscine species.

**Ultimobranchial Gland - Problems Encountered Experimentally**

As previously noted, there can be little question that the Corpuscles of Stannius have an effect upon calcium metabolism in bony fishes. The role of the ultimobranchial gland and its hormone calcitonin, is less clear. A number of investigators (e.g. Pang & Pickford, 1967; Pang, 1971b; Lopez, et al., 1971) were unable to elicit hypocalcemic responses in a variety of teleosts using both mammalian and piscine calcitonin preparations. In other studies administration of calcitonin did prompt reductions in the plasma calcium levels of eels (e.g. Chan, et al., 1968; Pang, 1971b; Lopez, et al., 1976). Even in this species, however, such treatment prompted erratic responses (Chan, 1972; Pang, 1971b; Orimo, et al., 1972). A serious problem of studies of this kind stems from the difficulties encountered in
attempts to totally remove the ultimobranchial gland surgically, for these, almost inevitably, lead to serious damage to the test animal. Thus, reliable ablation-replacement therapy studies are virtually non-existent. Consequently, failure to evoke plasmic responses to administered calcitonin may actually reflect little more than the fact that the normal intact animal already has adequate amounts of the hormone present. Finally, while such studies are equivocal, it is apparent that calcitonin does prompt marked alterations in the bone of eel (Lopez, et al., 1976) and presumably this can be related to calcium metabolism.

Finally, it may be noted that calcitonin involvements in reproductive functions have been observed (Oguri, 1973; Deftos, et al., 1974; Orimo, et al., 1977; Yamane & Yamada, 1977). Calcitonin influence may well be more extensive than is presently appreciated. Nevertheless, there is acceptable evidence suggesting an ultimobranchial role in calcium metabolism.
MATERIALS AND METHODS

Maintenance and Conditions of Acclimation

Goldfish (Carassius auratus, variety-common) were obtained from Hartz Mountain, St. Thomas, Ontario, in the late autumn and winter and were randomly distributed in groups of twenty among seven, two-hundred litre fiberglass tanks of a neutral, non-reflecting green color. Each tank was equipped with a high-capacity filtration system (approx 5 L min⁻¹) and a stainless steel 500 W immersion heater linked to a thermistor-relay system. Temperatures of 30 ± 1°C (mean ± maximum departure from mean) were maintained and no significant temperature differences were recorded among tanks. A photoperiod hood with an Intermatic T101 time switch ensured photoperiods accurate to about ± 10 minutes. These were used to control 15 W incandescent shielded bulbs which gave water surface light intensities ranging from 118 to 194 lux over individual tank surfaces. The photoperiod hoods were of a neutral, non-reflecting grey color.

Water quality parameters were periodically recorded. Free chlorine levels were negligible. Oxygen concentrations exceeded 80% saturation. Water hardness (CaCO₃) ranged from 135 to 145 mg L⁻¹. Total alkalinity (CaCO₃) ranged from 91 to 96 mg L⁻¹. pH varied from 7.4 to 7.8.

Specimens were fed Purina Fish Chow once daily, and after thirty minutes excess food and fecal materials were syphoned from the tank bottoms. Water levels were corrected daily for
loss due to evaporation with dechlorinated and aged water.

Throughout the period of study, test animals remained in an apparently healthy condition as judged by their general activity, feeding behaviour and absence of obvious disease symptoms. Most of the fish weighed between 8 and 15 gms and were between 7 to 10 cms in length and so could be categorized as a reasonably uniform group of small goldfish. Seven photo-period regimes were used in the study: OL:24D, 2L:22D, 4L:20D, 8L:16D, 12L:12D, 16L:8D, 24L:0D. Test groups were acclimated to their respective photoperiods for not less than 8 weeks prior to sampling. All light periods were initiated at 0800 hours and samples were taken between 0930 and 1030 hours.

**Blood Sampling : Plasma Calcium Determination**

Test animals were netted and stunned by a blow to the head. Ammonium heparin-treated 1 ml tuberculin syringes were used to take blood samples by caudal vessel puncture. Following centrifugation (5000 rpm, 5 min) plasma was drawn off by Pasteur pipette and stored in capped, polystyrene, disposable centrifuge tubes at -70°C prior to analysis (Kelvinator Series 100 Ultracold). Earlier studies (Smeda, pers. comm.) have shown that it is possible to store plasma samples in this manner for up to one year without significant changes in Ca++ concentration. Hemolyzed samples were discarded.

A microtitrimetric procedure was used to estimate plasma
calcium (Oxford Titrator Reagent Set). Titrations were done with disodium ethylene diamine tetracetate (EDTA) in the presence of a calcein indicator. Calcein complexes with calcium under alkaline condition (pH 12) to form a compound which fluoresces under ultra-violet light. EDTA chelates calcium and ultimately withdraws it from the complex. Therefore, the endpoint of titration is the disappearance of fluorescence. Possible interference by magnesium is prevented by the performance of the titration under pH conditions which lead to precipitation of magnesium as the hydroxide.

All estimates were done in triplicate, each with 50 μl plasma samples. Dilutions of Versatol, (Warner Lambert Diagnostics) a stable, human plasma of known calcium content, were used as standards. Plasma calcium concentrations were expressed as mM L⁻¹. Plasma calcium concentration of males only and females only were plotted against photoperiod. Means, variance and standard deviations were determined from the data and 95% confidence intervals were established in order to define the range within which the population mean would be expected to lie.

Histological Procedures

General

Immediately following the isolation of plasma samples, the pituitary, ultimobranchial glands and Corpuscles of Stannius were dissected out and fixed in Bouin's Fixative
(Appendix 1) for 24 hours. Tissues were then descaled if necessary and trimmed. Tissues were stored in 70% ethanol, dehydrated to 95% ethanol through a graded ethanol series, cleared in terpineol and embedded in Tissue Prep (Fisher; M.P. 56.7°C). Embedded tissue was then sectioned on a rotary microtome and serial sections of all tissues were affixed to coded microscope slides with Mayer's Albumen (Appendix 5). Sections were stained and slides were coverslipped using Canada Balsam. All tissues were examined using a Leitz Orthoplan Microscope and photographs taken on Kodak Panatomix X or Kodachrome 64 film with an automatic camera attachment.

(a) Pituitary

Pituitary glands were sectioned at 3 μm and stained using a modification of the Cleveland-Wolfe method (Lee, 1937). In the modified procedure, which provides good differentiation of cell types in the pars distalis of the goldfish, Aniline Blue was omitted, Haematoxylin and Erythrosin staining times were increased to 10 and 45 minutes respectively and alcoholic rather than aqueous Orange G was used (Appendix 4). This technique permitted the distinction of acidophilic prolactin cells from the acidophilic STH cells, since the former appear brick-red while the latter stain orange-red. Detailed histological examinations of glands from each photoperiod were carried out. Serial sections from specimens held under the various photoperiod regimes were examined to determine
orientation and plane of section through the gland. Because of the variable morphology of individual pituitary glands, some difficulty had been encountered in orienting them during embedding. Glands which had been sectioned with close to perfect frontal orientation were then chosen for analysis by means of the Quantimet 720 Computer Image Analyzer and, as a preliminary step, a detailed examination of one pituitary (♀, 8.20 gms 8L:16D) was carried out. Two columns of tissue extending vertically through the entire gland were analyzed on a section-by-section basis. Of these, one passed through a well vascularized area of the rostral pars distalis, whereas the other column extended through an area with little vascularization. In the analysis of these tissue columns, care was taken to ensure that the areas evaluated from successive serial sections included cells contiguous with and immediately subjacent to those of the preceding section. The aim of detailed examination will be apparent. It was considered essential to establish whether or not the region of particular interest, the rostral pars distalis, was internally homogeneous. Information of this kind is critically important in relation to sampling for assessment of photoperiod related variations in apparent activity. Of particular importance was the relation to vascularity in different regions of the RPD. In addition to the examination of the two 183 μm cores of tissue, six additional transects, each of which was comprised of approximately four 5000 μm² machine fields were
taken between the original tissue columns. These made possible examination of cells occupying the area between the two columns. The initial and final sections of the transects fell approximately into the region of the original tissue cores and consequently provided a basis for checking the accuracy of the analyses conducted on the principal core sections. Using the information provided by these preliminary investigations, single, adequate tissue columns representing appropriate areas were analyzed from pituitaries from the remaining photoperiod groups. These columns (90 \( \mu m \) to 132 \( \mu m \)) extended dorsoventrally through the rostral pars distalis. Individual sections were analyzed for prolactin cell numbers, total cytoplasmic plus nuclear areas and total nuclear area in a constant field of 5000 \( \mu m^2 \). From these values, mean nuclear area, cytoplasmic area, cytoplasmic to nuclear ratio and total cell areas were calculated for the initially analyzed pituitaries. Only C/N ratios were calculated for remaining photoperiods. It is appreciated that measures of volume rather than ratio are more appropriate for studies of this kind. However, Underwood (1968) has considered this question in some depth and concluded that the use of area rather than volume would be justifiable when the study populations were ostensibly isotrophic and approach isodimensionality. These conditions were at least approximated in the present study. Under such conditions, in a field containing approximately 150-200 cells, the projected cytoplasmic-to-nuclear area should approximate the corresponding
volume ratio. Even in the case of marked anisotrophy, ratios of area remain valid for parallel sections. Statistical treatment of the data was based upon procedures outlined by Bliss (1967, 1970). Tests were carried out using the Wang 2200 desk top computer.

For the initial pituitary examined, data for each parameter (C/N ratio, total cell area, cytoplasmic area, nuclear area) were linearly regressed against serial section (i.e. distance). Coefficients of determination (R) and correlation (r) were estimated. The significance of the latter were determined at the 0.05 level using critical values tabulated by Snedecor (1953). In no instance was a significant correlation demonstrated. However, when the C/N ratios were plotted against section number, they tended to "cluster" in alternative fashion above and below the least squares line-of-best fit. One example of this can be seen in Figure 8a (p.87) of the Results section. This kind of data distribution approximates a cyclical pattern and is not well described by a linear relationship. Consequently, an attempt was made to fit, by an essentially empirical method, a more appropriate trend line. The technique used for this was as follows. A trend line was first placed through these points by visual approximation. The deviations of the individual data points from this trend line were then plotted against section number. Least squares analysis was carried out on these departures to minimize total deviation and the originally-placed trend line
accordingly adjusted. Each new "best-fit-curve" was then adjusted until minimum variation and standard deviation were achieved.

This approach appeared to represent the least objectionable method of generating a descriptive relation for a series of values of irregular amplitude and period and thus was not easily described by the normal periodic regression techniques outlined by Bliss (1970).

All subsequent pituitary samples from the various photoperiod groups were accordingly analyzed in a similar fashion. Mean cytoplasmic and nuclear areas of cells from peak, trough, upslope and downslope regions of the resultant C/N ratio wave pattern were then estimated for comparative purposes.

(b) **Corpuscles of Stannius**

Corpuscles of Stannius were serially cross-sectioned with care taken to insure that tissue columns ran as closely as possible along the anterior-posterior axis.

These were stained with Haematoxylin and Eosin (Appendix 2 and 3).

As with the pituitary samples, one corpuscle ($\sigma^1$, 11.7 g, 12L:12D) was initially sectioned (3 μm) throughout to assess the internal homogeneity of the gland. For each of the 61 resulting sections (sections 1-29 on one slide, sections 30-61 on a second slide), the epithelial cells occupying 5000 μm$^2$ fields on subjacent sections were evaluated by means of the
Quantimet for cell numbers, overall cytoplasmic plus nuclear areas and nuclear area. Mean cell area, nuclear area, cytoplasmic area and cytoplasmic:nuclear area ratio were then calculated. These data were treated as previously described. Each parameter was regressed against section position and coefficients of determination and correlation calculated. As some evidence of cyclical variation was observed, a trend line was again fitted by the means previously described.

On the basis of this analysis serial sections were cut from the remaining samples; one male and one female being considered from each photoperiod regime other than OL:24D. In the latter instance a single male was examined. For the most part tissue columns including at least 8 to 10 sections (3 μm) were sectioned from the mid-to-posterior region of the corpuscle, the area in which least variability was encountered. In some instances, however, 7 μm sections were also examined. This was done primarily to evaluate the effect of section thickness upon the discriminatory capacity of the Quantimet. A total of 14 specimens was therefore examined to estimate the effects of photoperiod on these animals.

(c) Ultimobranchial Glands

Ultimobranchial glands were sectioned at 7 μm and stained with Haematoxylin and Eosin (Appendix 2,3). Histological examination of the glands revealed two basically different cell
forms; one with clear cytoplasm and the other with eosinophilic to basophilic cytoplasm. Approximately one thousand cells from an average of at least three different individuals of either sex from each photoperiod were counted and the percentages of clear versus eosinophilic or basophilic cells determined. Differences in the appearance of epithelia at all photoperiods were also recorded.
RESULTS

Plasma Calcium

Figure 3 summarizes variations in plasma calcium levels in test animals held at the different photoperiod regimes. Because of known sexual dimorphism in relation to calcium metabolism in teleosts, males and females were considered separately. Relatively few animals were sampled under conditions of total darkness and total light. Consequently some dispersion is present in these values and the 95% confidence intervals of the mean commonly exceeded ranges in these samples.

Within these limitations, the data suggested that in female goldfish, plasma levels of calcium increased with lengthening light period and stabilized at approximately 2.5 mM/L to between photoperiods 8L:16D and 16L:8D. The extreme range of the confidence interval obtained under conditions of total light precluded any conclusion regarding trends in plasma calcium levels under these conditions. The same considerations apply to the male goldfish sampled following acclimation to total darkness and two hours of light, but between photoperiods of 4L:20D and 16L:8D plasma calcium was again stable at approximately 2.5 mM/L. Under

1. The 95% confidence interval of the mean was calculated as $X \pm S, t$. This interval defines, with a probability of 0.05, the range within which the population mean would be expected to lie.
Figure 3.

Plasma calcium (mM$^{-1}$) in male and female goldfish in relation to photoperiod. Horizontal line: mean, vertical line: range, vertical bar: 95% confidence interval of the mean, bracketed number: sample size.
conditions of continuous light, plasma calcium decreased by some 25% to 1.9 mM/L\(^{-1}\). Generally, over a substantial range of photoperiods (i.e. 8L:16D to 16D:8L) no significant differences in plasma calcium levels were apparent between the male and female goldfish. At 4L:16D calcium level (2.53 mM/L\(^{-1}\)) in the male exceeded that (2.34 mM/L\(^{-1}\)) in the female.

The most notable feature of these results was the absence of any marked variation in plasma calcium levels over what might be regarded as the 'normal' range of photoperiods in the north temperature zone, i.e. 4-6L:18-20D to 16L:8D.
Pituitary

As noted under the heading, Review of Literature, three main regions are distinguishable in the pituitary gland of C. auratus; the pars nervosa (PN), pars intermedia (PI) and pars distalis (PD). The latter can be further subdivided into a proximal pars distalis (PPD) and a rostral pars distalis (RPD), (Figure 4). Although the goldfish pituitaries examined displayed considerable variability in overall shape, the rostral pars distalis normally formed a "U"-shaped band, the central part of which comprised the anterior portion of the pars distalis while the arms of the "U" extended laterally along the sides of the distalis toward the infundibular stalk. The central or basal portions of the band were substantially thicker than the arms. There was little extension of the RPD tissue over the dorsal or ventral portions of the proximal pars distalis. Previous descriptions of the gross morphology of the pituitary of this species are largely confirmed in the present study.

General Histology of the Rostral Pars Distalis

Rostral tissue consisted primarily of brick red-staining prolactin cells. Interspersed among these were chromophobic and more rarely basophilic cells. On the basis of earlier reports, considered in Review of Literature, the latter were tentatively identified as ACTH and TSH-secreting cells
Figure 4. Section through the pituitary to illustrate the well-defined rostral pars distalis (RPD) composed primarily of prolactin cells, the proximal pars distalis (PPD), pars intermedia (PI) and pars nervosa (PN). Note the well vascularized area of the RPD in which chromophobes surround the vessels and produce the lighter patches (pointers). Areas inside the circles approximate the groups of cells analyzed from serial sections in vascular (anterior circle) regions. Mag. X 300.
respectively. The anterior areas were vascularized by both capillaries and sinuses. These vessels were frequently surrounded by chromophobes. Chromophobes also occurred individually and in small aggregations among the prolactin cells which formed the bulk of the tissue mass (Figure 4). Superficially, prolactin cells appeared to be arranged in discrete cords and clumps. It is the blood vessels, sinuses, connective tissue, basophils and chromophobes within this matrix of prolactin cells that produce the superficial discontinuity noted on single sections of this tissue. Examination of adjacent serial sections indicated that these cells may form a continuous mass. These observations support the conclusions of Leatherland (1972), rather than earlier descriptions by Bell (1938). Although the adjacent proximal pars distalis included acidophilic cells, tentatively STH-secreting cells, they were distinguished from the prolactin cells by their affinity for Orange G rather than Erythrosin in the Cleveland-Wolfe stain. The prolactin cell cytoplasm stained brick-red and was finely granular, whereas that of the STH cells stained pink or orange and had a homogeneous rather than a granular appearance.

Initial Analysis of one Gland for Internal Pituitary Organization

As noted in Materials and Methods, one pituitary was analyzed in detail, using the Quantimet 720, to assess the
extent of internal organizational homogeneity and so provide
a basis for subsequent sampling. This was taken from a
test animal maintained under the 8L:16D photoperiod regime.
Serial sections were analyzed for prolactin cell number, total
cytoplasmic plus nuclear area and total nuclear area in
constant machine fields of 5000 \( \mu m^2 \). From these values,
average nuclear area, average cytoplasmic area, average
cytoplasmic-to-nuclear ratio and average cell areas were
calculated for the two columns of tissue analyzed. The less
vascularized area from which one core was taken will be
referred to hereafter as the avascular region, while the
vascularized area from which the second core was taken will
be referred to as the vascularized region.

Avascular Region

In Figure 5, average nuclear area has been plotted
against serial section number throughout the 183 \( \mu m \) core of
tissue. Regression of nuclear area against section position
led to a slope value which approached zero. The correlation
coefficient was not significant at \( p = 0.05 \) (Table 2).
Similarly, regressions of average cytoplasmic area, average
cell area and average cytoplasmic : nuclear area ratio
against section position were characterized by slopes close
to zero and correlation coefficients which were not significant
at the 0.05 level (Figures 6, 7 and 8a).
Figure 5. Regression of average nuclear area with serial section number. Avascular region. 8L:16D.
Figure 6. Regression of average cytoplasmic area with serial section number.
Avascular region. 8L:10D.
Figure 8a. Regression of C/N ratio with serial section number. Avascular region, 8L:16D.

Figure 8b. Regression of C/N ratio with serial section number. Vascular region, 8L:16D.
In Figure 9, average nuclear area has been plotted against serial section number through the corresponding 183 μm core of tissue from the vascularized area. Regression of nuclear area against section position led to a value for the slope which approached zero. The correlation coefficient was not significant at p = 0.05 (Table 2). Similar treatment of values for average cytoplasmic area, average cell area and cytoplasmic-to-nuclear area lead to similar findings. The slopes of linear regression equations did not differ significantly from zero and all correlation coefficients were non-significant (Figures 10, 11 and 8b). The statistical information for average cytoplasmic, nuclear and cell areas and C/N ratio is summarized in Table 2 for both series of serial sections. Although these observations suggest histological homogeneity, further examination of the data suggested that, in some cases, (e.g. Figures 8a and 8b) points were distributed in "groups" or "clusters" alternately above and below their least squares lines-of-best fit. This suggested the possibility of some form of cyclical morphological variation along the tissue cores. Accordingly, each parameter was examined in more detail to assess this possibility.

Nuclear area - The distribution of values describing nuclear area in the vascularized region did not indicate any readily
Figure 9. Regression of average nuclear area with serial section number.
### TABLE 2

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>LINEAR REGRESSION Equation (y=ax+b)</th>
<th>( R^2 )</th>
<th>( r^2 )</th>
<th>Significance ***</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Avasc. Tissue Core</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear Area</td>
<td>( y = 0.0086X + 18.1 )</td>
<td>0.0028</td>
<td>0.0530</td>
<td>NS</td>
</tr>
<tr>
<td>Cyto Area</td>
<td>( y = 0.0206X + 14.5 )</td>
<td>0.0106</td>
<td>0.1031</td>
<td>NS</td>
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<tr>
<td>Cell Area</td>
<td>( y = 0.0273X + 32.7 )</td>
<td>0.0427</td>
<td>0.2066</td>
<td>NS</td>
</tr>
<tr>
<td>Ratio</td>
<td>( y = 0.0013X + 0.83 )</td>
<td>0.0110</td>
<td>0.1047</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Vasc. Tissue Core</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear Area</td>
<td>( y = 0.0358X + 16.6 )</td>
<td>0.0376</td>
<td>0.1939</td>
<td>NS</td>
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<td>Cyto Area</td>
<td>( y = 0.0115X + 12.9 )</td>
<td>0.0036</td>
<td>0.0601</td>
<td>NS</td>
</tr>
<tr>
<td>Cell Area</td>
<td>( y = 0.0396X + 29.7 )</td>
<td>0.0355</td>
<td>0.1883</td>
<td>NS</td>
</tr>
<tr>
<td>Ratio</td>
<td>( y = 0.0013X + 0.70 )</td>
<td>0.046</td>
<td>0.1206</td>
<td>NS</td>
</tr>
</tbody>
</table>

*\( R \), coefficient of determination

**\( r \), coefficient of correlation

***NS, not significant at \( P = 0.05 \)

discernible pattern of fluctuation (Figure 9). By contrast, those from the avascularized region gave the impression of an irregular pattern consisting of two waves (Figure 5).

**Cytoplasmic area** - The distribution of points describing changes in cytoplasmic area through the vascularized region suggested a pattern of two and possibly three irregular waves (Figure 10). That for the avascularized region was strongly...
Figure 10. Regression of average cytoplasmic area with serial section number.
suggestive of a three wave pattern (Figure 6).

**Cell Area** - Distribution of cell area values from the vascularized region suggested a pattern consisting of two readily discernible, low amplitude waves (Figure 11). Distributions from the avascularized region took the form of two irregular, high amplitude wavelengths (Figure 7).

**C/N Ratio** - The distribution of values for the vascularized region suggested a fairly regular cycle in C/N ratio values composed of relatively low amplitude wavelengths (Figure 8b). In the avascularized region, a more regular pattern, composed of four high amplitude wavelengths was apparent (Figure 8a). Therefore, in many instances, there was at least some evidence of the presence of wavelike patterns in the distribution of the data points. The pattern was most clearly demonstrated in the case of C/N ratio, wherein the distribution of the data points suggested the presence of four complete wavelengths.

An attempt was made to fit curves through these data by means of the periodic regression procedures outlined in Bliss (1970). The simplest of the relations expressed by these techniques takes the form of a symmetrical sinewave extended by Fourier terms. The line-of-best fit through values for C/N ratio, which resulted from the application of this method, proved to be an obviously inadequate description of the data, for the apparent waveforms were not constant in period or
Figure 11. Regression of average cell area with serial section number. Vascular region. 8L:16D.
amplitude. Thus, a simple sinewave pattern does not account for the observed variations in amplitude and period within the tissue cores.

Since neither linear nor periodic regressions were felt to adequately describe the variations observed, a third approach was used in an attempt to describe the wave-like distribution of C/N ratio values. In this instance, a "trend" line was first visually approximated through the data. This was subsequently adjusted by the least squares approach previously described. The resulting "trend" line through the C/N ratio data set for the avascular tissue core is indicated in Figure 12a. The corresponding "trend" line through the data set for the tissue core from the vascularized region is shown in Figure 12b. A period of approximately 36 $\mu$m was apparent in both tissue cores. Amplitudes of approximately 0.80 and approximately 0.40 ratio units were observed in the avascular and vascular regions respectively.

Initial section-by-section examination of histological material had not revealed any strikingly obvious periodic differences in cellular morphology. However, examinations guided by reference to the C/N ratio waveforms did reveal several differences in morphological characteristics. These differences suggested the likelihood of variations in cellular activity. The following descriptions are based on the microscopic characteristics typical of cells lying within
Figure 12a. Curve through the C/N ratios of prolactin cells plotted against serial section number. Avascular region. 8L:16D.

Figure 12b. Curve through the C/N ratios of prolactin cells plotted against serial section number. Vascula Region. 8L:16D.
regions. The tissue cores' C/N ratios corresponded to the peak, trough, upslope and downslope portions making up the waveform.

**Peak Regions** - Cells from peak areas (Figure 13) possessed round to oval or kidney-shaped nuclei. Their moderate to plentiful brick-red cytoplasm appeared to be granular in nature. On the basis of the studies noted in Review of Literature, this may have been secretory product. Chromatin was diffuse. No prominent nucleoli were observed. Average cytoplasmic and nuclear areas were $16 \pm 7 \ \mu m^2$ and $16 \pm 4 \ \mu m^2$ and $16 \pm 2 \ \mu m^2$ and $16 \pm 2 \ \mu m^2$ for the avascular and vascularized regions respectively (Table 3).

**Trough Regions** - Prolactin cells in trough regions (Figure 4) also had round to oval nuclei with fine chromatin. Their nucleoli, however, were more prominent. Cytoplasm was scant and slightly basophilic in cast. It was also less granular in appearance. Average cytoplasmic and nuclear areas were $12 \pm 4 \ \mu m^2$ and $21 \pm 7 \ \mu m^2$ and $11 \pm 4 \ \mu m^2$ and $19 \pm 7 \ \mu m^2$ for avascular and vascularized regions respectively, (Table 3).

**Upslope Regions** - Cells in upslope regions (Figure 15), possessed round to oval nuclei with fine chromatin. Nucleoli were observed more frequently in the nuclei of cells in the lower upslope regions than in those cells occurring higher on the slope. Similarly, cytoplasm was less abundant in cell
Figure 13. Prolactin cells (e) of a peak area with abundant cytoplasm. Notice round, oval or kidney-shaped nuclei (pointer) characteristic of this stage. Mag. X 850.

Figure 14. Prolactin cells (e) of a trough area in which most cells exhibit very little cytoplasm. Notice round, bare-looking nuclei. The grey material (pointers) around the cells is connective tissue or ground substance and not cytoplasm. Mag. X 850.
Figure 15. Prolactin cells (e) of an upslope area in which most cells have a moderate amount of cytoplasm and round and oval nuclei. Mag. X 850.

Figure 16. Prolactin cells (e) of a downslope area in which most cells have a moderate amount of cytoplasm. Notice one of the kidney-shaped nuclei (pointer) characteristic of this stage. Mag. X 850.
groups examined from low upslope regions than in groups examined from higher up the slope and was also less granular in appearance. Average cytoplasmic and nuclear areas were $13 \pm 5 \, \mu m^2$ and $19 \pm 5 \, \mu m^2$ and $11 \pm 3 \, \mu m^2$ and $18 \pm 4 \, \mu m^2$ for avascular and vascularized regions respectively (Table 3).

Downslope Regions - Prolactin cells in downslope regions (Figure 16) were more variable in appearance than those of the other regions. Nuclei from all groups higher up on the slope were round or oval-shaped. However, lower down the slope, kidney-shaped nuclei and, infrequently two small nuclei per cell were observed also. Chromatin was diffuse although some clumping was evident often. In near-trough regions the frequency of occurrence of nucleoli increased. Cytoplasm was more abundant in cell groups higher on the slope and decreased progressively down the slope. Cytoplasmic and nuclear areas were $13 \pm 5 \, \mu m^2$ and $16 \pm 6 \, \mu m^2$ and $12 \pm 4 \, \mu m^2$ and $17 \pm 3 \, \mu m^2$ for avascular and vascularized regions respectively (Table 3).

TABLE 3.

Mean Cytoplasmic and Nuclear Areas for Cells from Peak, Trough, Upslope and Downslope Regions of the C/N ratio produced Wave pattern.

<table>
<thead>
<tr>
<th>REGION</th>
<th>CYTOPLASMIC AREA, $\mu m^2$</th>
<th>NUCLEAR AREA, $\mu m^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avascular Region</td>
<td>Vascular Region</td>
</tr>
<tr>
<td></td>
<td>$\bar{X}$ R S.E. $\bar{X}$ R S.E. $\bar{X}$ R S.E. $\bar{X}$ R S.E.</td>
<td></td>
</tr>
<tr>
<td>Peak</td>
<td>$16 \pm 7$ .86 $16 \pm 2$ .72 $16 \pm 7$ .86 $16 \pm 2$ .52</td>
<td></td>
</tr>
<tr>
<td>Trough</td>
<td>$12 \pm 4$ .80 $11 \pm 4$ .73 $21 \pm 7$ .97 $19 \pm 7$ 1.09</td>
<td></td>
</tr>
<tr>
<td>Upslope</td>
<td>$13 \pm 5$ 1.48 $11 \pm 3$ .62 $19 \pm 5$ 1.01 $18 \pm 4$ .64</td>
<td></td>
</tr>
<tr>
<td>Downslope</td>
<td>$13 \pm 5$ 1.48 $12 \pm 4$ .67 $16 \pm 6$ 1.26 $17 \pm 3$ .60</td>
<td></td>
</tr>
</tbody>
</table>

$\bar{X}$ - mean.  R - range.  S.E. standard error.
Transects including the original tissue areas were next examined and all cells lying within the 150 μm distance between the columns analyzed in a similar manner using the Quantimet 720. This was done in an attempt to determine whether cells in these "intercolumn" regions were morphologically similar to the column cells and to assess whether or not they too provided evidence of cyclical variation in appearance. As the original C/N ratio co-ordinates had not been recorded, the transects could only be approximately positioned. An attempt was made, however, to include areas as close to the original columns as possible, the tissue lying in a plane perpendicular to these areas and usually regions peripheral to the originally analyzed areas. It was also not possible to ensure the precise alignment of consecutively analyzed areas (machine fields) of the tissue because of difficulties in determining boundaries. The positions of the six transects examined are indicated in Figure 17. All transects extended from the avascular tissue core to the vascularized tissue core. The C/N ratios of these cells appear in Table 4. Transect number 1 (Table 4), and Figure 17 extended from a trough in the avascular region which corresponded to a trough in the vascular region. C/N ratio values for the transect indicated that the transect began in a region of trough values (0.58) extended into a region of intermediate values (0.87 and 0.88) and
Figure 17. Results of transect analysis performed on selected sections across the areas originally analyzed. 8L:16D.
TABLE 4.

C/N Ratio Values for Cells Located along the Transects Between the Avascular and Vascular Tissue Cores

<table>
<thead>
<tr>
<th>TRANSECT FIELD NO.</th>
<th>TRANSECT NUMBER</th>
<th>(Serial Section Number)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1(3)</td>
<td>1(10)</td>
</tr>
<tr>
<td>1</td>
<td>0.58</td>
<td>0.85</td>
</tr>
<tr>
<td>2</td>
<td>0.87</td>
<td>1.01</td>
</tr>
<tr>
<td>3</td>
<td>0.88</td>
<td>0.77</td>
</tr>
<tr>
<td>4</td>
<td>1.07</td>
<td>0.71</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

terminated on a region of peak values (1.07). The initial value (0.58) from the transect coincided well with the expected value of 0.60 for the trough of the avascular region but the final transect reading of 1.08 did not coincide with the expected trough value (0.45) obtained from initial core analysis. Transect number 2 (Table 4 and Figure 17) extended from an avascular upslope region which corresponded to a vascular upslope area. C/N ratio values indicated that the transect began in a region of upslope values (0.85), may have extended into a region of peak values (1.01) or at least into a higher upslope region and gradually lowered on a region of upslope values (0.77 and 0.71). The initial transect value of 0.85 coincided almost exactly with the
expected value (0.82) for the upslope. The final transect value (0.71) was, however, slightly lower than the value (0.86) generated on initial core analysis. Transect number 3 extended from a mid-upslope region of the avascular core to a high peak in the vascular region. C/N ratio values indicated that the transect began in a region of upslope values (0.88) and progressively approached peak values (0.94 to 1.07). This was followed by a slight decrease (0.86) and terminated on a region of peak values (1.25). The initial transect value (0.88) differed from the expected value of 1.17 while the final transect reading (1.25) was much higher than the expected peak value (0.81) obtained from initial core analysis. Transect number 4 extended from a low-upslope area from the avascular tissue core to a mid-upslope area in the vascular region. C/N ratio values indicated that the transect began in a region of high upslope values (0.83) and this slope progressively decreased (0.81 to 0.60) to terminate in a region of trough values (0.32). The initial value of the transect (0.83) did not coincide with the expected value (0.48) while the final transect value (0.32) was much lower than that expected (0.78) from original core analysis. Transect number 5 extended from a mid-upslope region of the avascular tissue core to a peak in the vascular region. C/N ratio values indicated that the transect began in a region of mid-upslope values (0.93), was followed by a
slight decline of the slope (0.85) and subsequently entered into a region of peak values (1.03). The initial value from the transect (0.93) coincided closely with the expected value of 0.96 while the final transect reading (1.03) was slightly higher than the expected peak value of 0.95 obtained from initial core analysis. Transect number 6 extended from a low downslope from the avascular region to a trough in the vascular region. C/N ratio values indicated that the transect began in a region of low downslope values (0.69) and maintained generally the same values (0.71 and 0.72) and terminated in a region of low-downslope values. The initial transect value (0.69) was much lower than the expected value (1.12) for a high upslope value from the original tissue core, while the final transect value (0.72) was higher than the expected value (0.59) from the initial core analysis for the vascularized region.

Tissue Core Analysis from the Vascularized Region of the Rostral Pars Distalis for Test Animals from other Photoperiods

24L:0D (Q, 21.68 gms) - C/N ratio values were linearly regressed against serial section number through a 72 μm core of tissue. No significant (p = 0.05) correlation was found (Table 7). The line of best fit through these data was placed by previously described methods and appears in Figure 18. A periodicity of approximately 30 μm was evident, as
Figure 18. Curve through the C/N ratios of prolactin cells plotted against serial section number. Vascular region. 24L:0D. The period is approximately 30 μm and the amplitude is approximately 0.20 ratio units.
was an amplitude of approximately 0.20 C/N ratio units.

16L:8D (♀, 9.50 gms) - When C/N ratio values were regressed against serial section number through a 66 µm column of tissue a barely significant (p < 0.05) correlation was obtained (Table 7). The regression equation (\( y = 0.0102X + 0.6814 \)) accounted for 20.8% of the total variance.

A periodicity of approximately 15 µm was evident for the curved "trend" line placed through the data set. The amplitudes for all waves was approximately 0.30 units (Figure 19).

12L:12D (♂, 7.28 gms) - C/N ratio values were linearly regressed against serial section number for a 123 µm column of tissue. No significant (p = 0.05) correlation was found (Table 7). A "trend" line was placed through these data and subsequently adjusted by the least squares method previously described. This curve appears in Figure 20. A decreasing period for these wave patterns was evident and ranged from 51 µm to 33 µm to 21 µm. Similarly, a decrease in the amplitude (0.40 units) between the first wave (sections 5-22) and the remaining waves (0.30 units; sections 22 to 33 and sections 33 to 40) occurred.

4L:2OD (♂, 9.76 gms) - When C/N ratio values were linearly regressed against serial sections through a 93 µm core of tissue, a barely significant (p ≤ 0.05) correlation was found (Table 7). The regression in this case (\( y = -1.0060X + \))
Figure 19. Curve through the C/N ratios of prolactin cells plotted against serial section number. Vascular region. 16L:8D. The periods of the wave-like curves are 15 μm. The amplitudes are approximately 0.30.
Figure 20. Curve through the C/N ratios of prolactin cells plotted against serial section number. Vascular region. 12L:12D.
accounted for 13.1% of the variance.

The curved trend line through these data appears in Figure 21. A decreasing periodicity (33 μm to 27 μm to 15 μm) was apparent. An amplitude of approximately 0.30 C/N ratio units was noted.

OL:24D (♀, 19.19 gms) - C/N ratio volumes were linearly regressed against serial section number for a 81 μm core of tissue. No significant (p = 0.05) correlation was found (Table 7). The trend line through these data points appears in Figure 22. No estimate of period could be made in this case. However, C/N ratio values lay within approximately a 0.30 unit range.

Average cytoplasmic and nuclear areas for cells from peak, trough, upslope and downslope regions of the C/N ratio produced wave patterns from the pituitary glands of the test animals from the remaining photoperiods are shown in Table 6.

### TABLE 5.

<table>
<thead>
<tr>
<th>PHOTO-</th>
<th>CORE</th>
<th>SEX</th>
<th>WEIGHT (gms)</th>
<th>PERIODICITY OF WAVE (μm)</th>
<th>APPROX AMPLITUDE OF WAVE (C/N ratio units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PERIOD</td>
<td>REGION</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OL:24D</td>
<td>Vasc.</td>
<td>♀</td>
<td>19.19</td>
<td>54 to 27</td>
<td>0.30</td>
</tr>
<tr>
<td>4L:20D</td>
<td>Vasc.</td>
<td>♂</td>
<td>9.76</td>
<td>33 to 27 to 15</td>
<td>0.30</td>
</tr>
<tr>
<td>8L:16D</td>
<td>Avasc.</td>
<td>♀</td>
<td>8.20</td>
<td>36</td>
<td>0.80</td>
</tr>
<tr>
<td>8L:16D</td>
<td>Vasc.</td>
<td>♀</td>
<td>8.20</td>
<td>36</td>
<td>0.40</td>
</tr>
</tbody>
</table>
Figure 21. Curve through the C/N ratios of prolactin cells plotted against serial section number. Vascular region. 4L:20D. Periods are 33 μm and 27 μm. The amplitudes are approximately 0.30.
Figure 22. Curve through the C/N ratios of prolactin cells plotted against serial section number. Vascular region. 0L:24D.
Table 5 summarizes data pertaining to the C/N ratio trend line of best fit for the test animals at all photoperiods.
TABLE 5. (continued)

Summary of Data for C/N Ratio Trend Line of Best Fit for all Test Animals Held Under Various Photoperiod Conditions

<table>
<thead>
<tr>
<th>PHOTO-PERIOD</th>
<th>CORE REGION</th>
<th>SEX</th>
<th>WEIGHT</th>
<th>FIG. PERIODICITY OF WAVE (µm)</th>
<th>APPROX. AMPLITUDE OF WAVE (C/N Ratio units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12L:12D</td>
<td>Vasc.</td>
<td>♂</td>
<td>7.28</td>
<td>51 to 33</td>
<td>0.40 to 0.30</td>
</tr>
<tr>
<td>16L:8D</td>
<td>Vasc.</td>
<td>♂</td>
<td>9.50</td>
<td>15</td>
<td>0.20 to 0.30</td>
</tr>
<tr>
<td>24L:0D</td>
<td>Vasc.</td>
<td>♀</td>
<td>21.68</td>
<td>30</td>
<td>0.20</td>
</tr>
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</table>

TABLE 6.

Cytoplasmic and Nuclear Areas for Cells from Peak, Trough, Upslope and Downslope Regions of the C/N Ratio Produced Wave Patterns.

<table>
<thead>
<tr>
<th>PHOTOPERIOD</th>
<th>REGION</th>
<th>CYTOPLASM</th>
<th>NUCLEUS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AREA - µm²</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>X</td>
<td>R</td>
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<tr>
<td></td>
<td></td>
<td>X</td>
<td>R</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>PHOTOPERIOD</th>
<th>REGION</th>
<th>CYTOPLASM</th>
<th>NUCLEUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>4L:20D</td>
<td>Peak</td>
<td>11 ± .5</td>
<td>.23</td>
</tr>
<tr>
<td></td>
<td>Trough</td>
<td>8 ± .5</td>
<td>.51</td>
</tr>
<tr>
<td></td>
<td>Upslope</td>
<td>10 ± 1</td>
<td>.54</td>
</tr>
<tr>
<td></td>
<td>Downslope</td>
<td>9 ± 1</td>
<td>.75</td>
</tr>
<tr>
<td>12L:12D</td>
<td>Peak</td>
<td>15 ± 2</td>
<td>.83</td>
</tr>
<tr>
<td></td>
<td>Trough</td>
<td>9 ± 2</td>
<td>.67</td>
</tr>
<tr>
<td></td>
<td>Upslope</td>
<td>12 ± 2</td>
<td>.76</td>
</tr>
<tr>
<td></td>
<td>Downslope</td>
<td>12 ± 2</td>
<td>.90</td>
</tr>
<tr>
<td>16L:8D</td>
<td>Peak</td>
<td>12 ± 3</td>
<td>1.23</td>
</tr>
<tr>
<td></td>
<td>Trough</td>
<td>8 ± 2</td>
<td>.65</td>
</tr>
<tr>
<td></td>
<td>Upslope</td>
<td>11 ± 1</td>
<td>.29</td>
</tr>
<tr>
<td></td>
<td>Downslope</td>
<td>13 ± 1</td>
<td>1.62</td>
</tr>
<tr>
<td>24L:0D</td>
<td>Peak</td>
<td>12 ± 1</td>
<td>.66</td>
</tr>
<tr>
<td></td>
<td>Trough</td>
<td>7 ± 1</td>
<td>.33</td>
</tr>
<tr>
<td></td>
<td>Upslope</td>
<td>14 ± 2</td>
<td>1.44</td>
</tr>
<tr>
<td></td>
<td>Downslope</td>
<td>14 ± 3</td>
<td>1.96</td>
</tr>
</tbody>
</table>

X - mean.
R - range.
S.E. - standard error.
<table>
<thead>
<tr>
<th>PHOTOPERIOD</th>
<th>PARAMETER</th>
<th>LINEAR EQUATION OF LINE</th>
<th>R*</th>
<th>r**</th>
<th>Significance***</th>
</tr>
</thead>
<tbody>
<tr>
<td>12L:12D</td>
<td>Ratio</td>
<td>y = 0.0041X + 0.8504</td>
<td>0.0539</td>
<td>0.2322</td>
<td>N.S.</td>
</tr>
<tr>
<td>16L:8D</td>
<td>Ratio</td>
<td>y = 0.0102X + 0.6814</td>
<td>0.2076</td>
<td>0.4557</td>
<td>S.</td>
</tr>
<tr>
<td>24L:0D</td>
<td>Ratio</td>
<td>y = 0.0004X + 0.5284</td>
<td>0.0027</td>
<td>0.0521</td>
<td>N.S.</td>
</tr>
<tr>
<td>0L:24D</td>
<td>Ratio</td>
<td>y = -0.0063X + 0.9367</td>
<td>0.1394</td>
<td>0.3733</td>
<td>N.S.</td>
</tr>
<tr>
<td>4L:20D</td>
<td>Ratio</td>
<td>y = -0.0060X + 0.5969</td>
<td>0.1309</td>
<td>0.3618</td>
<td>S.</td>
</tr>
</tbody>
</table>

R* coefficient of determination  
r** coefficient of correlation  
Significance*** at p = 0.05.
**Corpuscles of Stannius**

In *C. auratus*, the Corpuscles of Stannius are located dorsally and retroperitoneally in proximity to the mesonephritic ducts. These paired glands are oval in shape and are encapsuled with connective tissue which also penetrates and divides the gland into "tubules" and "pseudotubules". The observations of these glands in the present study are in agreement with descriptions by Krishnamurthy & Bern (1969), (Figure 23).

Examination of serial cross sections through many corpuscles suggested that the tubules and pseudotubules were oriented perpendicular to this plane of section and were aligned roughly parallel to the longitudinal axis of the gland. Well formed tubules were most frequently localized centrally, whereas peripheral tubules often tended to be randomly oriented. The polygon-shaped cells which lined the tubules were 6-10 μm in diameter. Each possessed a slightly eccentric round to oval nucleus approximately 4-6 μm in diameter. Capillaries, venules and possibly venous sinuses vascularized this tissue. Nerve cells and their processes were frequently encountered (Figure 24).

**Extensive Analysis of One Gland**

The initial corpuscle analyzed using the Quantimet 720 was from a test animal maintained on a 12L:12D photoperiod
Figure 23. Low power micrograph of the Corpuscles of Stannius showing pseudotubules. The rectangle represents the area analyzed by the Quantimet 720 from each cross-section. Mag. X 420.

Figure 24. A typical area of the corpuscle to show pseudotubules lined by secretory cells. Note the connective tissue sheath (ct) of the tubules and the open lumens (pointers) of tubules. Mag. X 1400.
regime. The gland was approximately 200 μm in length and 120 μm in diameter. Serial sections were analyzed for cell number, total cytoplasmic plus nuclear area and total nuclear area for epithelial cells occupying a constant machine field of 5000 μm². From these values, average nuclear area, average cytoplasmic area, average cytoplasmic to nuclear ratios and average cell areas were calculated for the 61 serial sections comprizing the centrally located core of tissue analyzed. In Figure 25, average nuclear area was plotted against serial section number. Regression analysis produced a value for the slope which approached zero. The correlation coefficient was significant \((p \leq 0.05, \text{Table 8})\). This, however, accounted for only 22% of the variance of this data set along the least squares line best fit. Examination of these data suggests that they occur in "clusters" alternating above and below the regression line. In all, four wave patterns were apparent. The regression of cytoplasmic area with serial section number (Figure 26) was characterized by a slope which approached zero. The correlation coefficient was significant \((p \leq 0.05, \text{Table 8})\). This least squares line-of-best-fit accounts for 28% of the observed variance. There was no obvious "clustering" of data in this graph as was apparent for the nuclear area plot. Any wave-like pattern may have been obscured by the small amplitude.
Figure 25. Regression of average nuclear area with serial section number.
Figure 26. Regression of cytoplasmic area with serial section number.
### TABLE 8.

Regression and Correlation Analysis for the Initial Corpuscle Analyzed

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>EQUATION OF THE LINE</th>
<th>$R^*$</th>
<th>$r^{**}$</th>
<th>Significance***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear Area</td>
<td>$y = ax + b$</td>
<td>0.2224</td>
<td>0.4716</td>
<td>S</td>
</tr>
<tr>
<td>Cytoplasmic Area</td>
<td>$y = 0.0849X + 14.07$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/N Ratio</td>
<td>$y = ax^b$</td>
<td>0.5428</td>
<td>0.7368</td>
<td>S</td>
</tr>
<tr>
<td>(all data pts.)</td>
<td>$y = 2.9413X^{-0.3173}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subset 1: Serials 1-12</td>
<td>$y = ax^b$</td>
<td>0.1380</td>
<td>0.3714</td>
<td>N.S.</td>
</tr>
<tr>
<td>Subset 2: Serials 13-28</td>
<td>$y = ax^b$</td>
<td>0.3035</td>
<td>0.5509</td>
<td>S</td>
</tr>
<tr>
<td>Subset 3: Serials 29-41</td>
<td>$y = ax + b$</td>
<td>0.1202</td>
<td>0.3467</td>
<td>N.S.</td>
</tr>
<tr>
<td>Subset 4: Serials 42-61</td>
<td>$y = ax^b$</td>
<td>0.0616</td>
<td>0.2481</td>
<td>N.S.</td>
</tr>
<tr>
<td>Total Aver. Cell Area</td>
<td>$y = ax + b$</td>
<td>0.0021</td>
<td>0.0460</td>
<td>N.S.</td>
</tr>
<tr>
<td>C/N Ratio Pts. 29-61</td>
<td>$y = ax + b$</td>
<td>0.0779</td>
<td>.2792</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

$R^*$, coefficient of determination
$r^{**}$, coefficient of correlation
S, N.S.*** significant or not significant at $P = 0.05$
Linear, power, exponential and logarithmic regressions of C/N ratio with serial section number were done for, (i) all data points, (ii) data point subsets, which included serial sections number 1 to 12, number 13 to 28, number 29 to 41 and number 42 to 61. A power curve best describes the line of best fit through the data set composed of 61 serial sections. The slope of the power function was -0.3173. The coefficient of correlation was significant (p ≤ 0.05, Table 8). Visual examination of C/N ratio values plotted against serial section number suggested that these data were divisible into 4 subsets. The lines of best fit through subsets 1, 2 and 4, which included serial sections number 1 to 12, 13 to 28 and 42 to 61 respectively, were best described by power curves. The slopes for these power functions were: -0.1509 for subset 1; 0.3677 for subset 2 and 0.0479 for subset 4. However, in all cases except that of subset 2, correlation coefficients were not significant (p = 0.05, Table 8). The line through subset 3, which included serial sections number 29 to 41, was best described by a linear equation. However, the coefficient of correlation was not significant (Table 8). C/N ratio values generated for the tissue from slide number 2 were linearly regressed with serial section number. No significant (p = 0.05) correlation was found (Table 8, Figures 27 and 28).

Regression of average cell area (Figure 29) with
Figure 27. Regression of C/N ratio, with serial section number for all data points.
Figure 28. Regression of C/N ratio data point subsets with serial section number.
section position (all sections) was characterized by a slope which approached zero. The correlation coefficient was not significant ($p = 0.05$, Table 8).

Examination of the points within this data set suggested that the points were distributed in "clusters" above and below the line of best fit. This indicated the possibility of some cyclical morphological variation along the tissue core. An empirically placed trend line was visually approximated through the average cell areas and is depicted in Figure 30. This then was adjusted by the method of least squares analysis previously described. The resulting curve had an approximate period of 42 $\mu$m and an approximate amplitude of 8 $\mu$m$^2$.

As previously stated, this tissue was contained on two different slides. An adjustment of 8 $\mu$m$^2$, which corresponded to a 30% difference in the average areas between the sections on these two slides, was made by raising the second slide accordingly. This adjustment resulted in the coincident continuation of an uninterrupted wave-like pattern.

Although initial section-by-section histological examination of this tissue revealed cells of variable morphology, no strikingly obvious periodic differences were noted. Re-examination of sections, directed by reference to the trend line placed through the cell area graph, provided a guide-line for the location of cells which might
Figure 29. Regression of average cell area with serial section number.
Figure 30. Curve through average cell area versus serial section number. The curve has an approximate period of 42 μm and an approximate amplitude of 8 μm². Cell area/volumes for sections 29 to 61 (slide 2) were raised by 8 μm² to fit with the curve from slide 1.
show differences in morphological characteristics that could be related to the activity of the cells. Descriptions are based on the microscopic appearance of the majority of cells in peak, trough, upslope or downslope regions of the wave-like trend line.

**Peak** - Cells from peak areas (Figure 31) typically possessed plump, round to oval or, less frequently, indented nuclei, which were basally to centrally located. No prominent nucleoli were observed in nuclei. Chromatin was diffuse and/or clumped. Basophilic cytoplasm was abundant. Average cytoplasmic and nuclear areas were $19.83 \pm 6 \mu m^2$ and $18.20 \pm 5 \mu m^2$ respectively. (Table 9).

**Trough** - Cells (Figure 32), had nuclei which were small and round through oval in appearance. Nucleoli were more commonly encountered in the nuclei of these cells. Chromatin was diffuse. Infrequently, cells that evidently were undergoing division and had 2 small nuclei were observed. Cytoplasm was basophilic and scant. Average cytoplasmic and nuclear areas were $16.28 \pm 5 \mu m^2$ and $15.65 \pm 3 \mu m^2$ respectively. (Table 9).

**Upslope** - Cells in upslope areas (Figure 33) had nuclei which were large and round to oval in shape. Nucleoli were prominent and sometimes numerous. Chromatin was diffuse.
Figure 31. A high power micrograph of cells from a peak area. Most cells are visibly larger than those from other regions of the wavepattern. Mag. X 1750.

Figure 32. A high power micrograph of cells from a trough area. Most cells are smaller than those from other regions of the wavepattern. Mag. X 1750.
Figure 33. A high power micrograph of cells from an upslope region showing cells intermediate in size between those from peak and trough regions. Mag. X 1750.

Figure 34. A high power micrograph of cells from a downslope region showing cells intermediate in size between those from peak and trough regions. Mag. X 1750.
Compared to trough cells, there was a slightly larger amount of basophilic cytoplasm. Average cytoplasmic and nuclear areas were $17.91 \pm 3 \mu m^2$ and $17.32 \pm 5 \mu m^2$ respectively, (Table 9).

**Downslope** - Cells in this region (Figure 34), typically possessed nuclei which were plump, round or oval to kidney-shaped. Nucleoli were present in some nuclei, but generally they occurred less frequently than in upslope regions. Chromatin was diffuse and/or clumped. Cytoplasm occurred in comparable amounts to that in upslope areas. Visually, the downslope and upslope regions were similar in appearance. The cytoplasmic and nuclear areas were $17.14 \pm 3 \mu m^2$ and $16.82 \pm 4 \mu m^2$ respectively (Table 9).

A series of photomicrographs were taken of the tissue analyzed in sections 29 to 46 in the cell area graph. These corresponded to approximately one wavelength. When these photographs were placed side by side, they documented well the changes in cell size which were suggested by the data obtained by Quantimet analysis.

**Tissue Core Analysis for the Corpuscles of Stannius from Test Animals from other Photoperiods**

Figure 27 suggested that C/N ratio volumes were less variable for the cells located in a more central region of the gland than those in a peripheral location. Therefore,
TABLE 9.

Average Cytoplasmic, Nuclear and Cell areas for Peaks, Troughs, Upslopes and Downslopes regions of the Cell Area Graph (Figure 30).

<table>
<thead>
<tr>
<th>REGION</th>
<th>CYTO. AREA ((\text{\mu m}^2))</th>
<th>NUCL. AREA ((\text{\mu m}^2))</th>
<th>CELL AREA ((\text{\mu m}^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\bar{x}) R</td>
<td>(\bar{x}) R</td>
<td>(\bar{x}) R</td>
</tr>
<tr>
<td>Peak</td>
<td>19.83 ± 6 .78</td>
<td>18.20 ± 5 .48</td>
<td>38.03 ± 2 .57</td>
</tr>
<tr>
<td>Trough</td>
<td>16.28 ± 5 .94</td>
<td>15.65 ± 3 .62</td>
<td>31.94 ± 3 .51</td>
</tr>
<tr>
<td>Upslope</td>
<td>17.91 ± 3 .71</td>
<td>17.32 ± 5 1.06</td>
<td>35.23 ± 3 .80</td>
</tr>
<tr>
<td>Downslope</td>
<td>17.14 ± 3 .97</td>
<td>16.82 ± 4 1.08</td>
<td>33.95 ± 3 .49</td>
</tr>
</tbody>
</table>

\(\bar{x}\) - mean; R - range; S.E. - standard error

for all test animals, a sample size of \(n = 10\) was taken from a central and comparable region within their corpuscles. This sample represented approximately 1500 cells and was considered adequate to provide information which would allow comparative analysis of morphological changes indicative of differences in cellular and possibly glandular activity which may be occurring between the animals. Regression and correlation analyses were performed on C/N ratio values versus serial section number for these cells. The results appear in Table 10. Comparison of the C/N ratio data were made by determining a mean ratio (Table 11), for each of the test animals. Mean C/N ratio values for males only showed an upward trend (0.78 to 1.18) in relation to the increasing number of light hours. Maximum ratio values occurred at 12L:12D (1.18) then decreased sharply. At 24L:0D the value (0.84) was similar to that at 0L:24D (0.78),
TABLE 10.

Regression and Correlation Analyses of C/N Ratio with Serial Section Number for all Test Animals at all Photoperiods

<table>
<thead>
<tr>
<th>PHOTOPERIOD</th>
<th>SEX</th>
<th>PARAMETER</th>
<th>EQ OF LINE</th>
<th>R*</th>
<th>r**</th>
<th>Significance***</th>
</tr>
</thead>
<tbody>
<tr>
<td>0L:24D</td>
<td>♂</td>
<td>C/N ratio</td>
<td>y = ax + b</td>
<td>0.3652</td>
<td>0.604</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>y = -0.0225X + 0.908</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2L:22D</td>
<td>♂</td>
<td>C/N ratio</td>
<td>y = -0.0131X + 0.9139</td>
<td>0.0480</td>
<td>0.2191</td>
<td>N.S.</td>
</tr>
<tr>
<td>2L:22D</td>
<td>♀</td>
<td>C/N ratio</td>
<td>y = -0.0095X + 0.9013</td>
<td>0.0238</td>
<td>0.1544</td>
<td>N.S.</td>
</tr>
<tr>
<td>4L:20D</td>
<td>♂</td>
<td>C/N ratio</td>
<td>y = 0.0279X + 0.6913</td>
<td>0.3037</td>
<td>0.5511</td>
<td>N.S.</td>
</tr>
<tr>
<td>4L:20D</td>
<td>♀</td>
<td>C/N ratio</td>
<td>y = 0.0084X + 0.8800</td>
<td>0.0169</td>
<td>0.1301</td>
<td>N.S.</td>
</tr>
<tr>
<td>8L:16D</td>
<td>♂</td>
<td>C/N ratio</td>
<td>y = 0.0028X + 0.9307</td>
<td>0.0075</td>
<td>0.0864</td>
<td>N.S.</td>
</tr>
<tr>
<td>8L:16D</td>
<td>♀</td>
<td>C/N ratio</td>
<td>y = 0.0071X + 0.9420</td>
<td>0.0257</td>
<td>0.1603</td>
<td>N.S.</td>
</tr>
<tr>
<td>8L:16D</td>
<td>♂</td>
<td>C/N ratio</td>
<td>y = 0.0098X + 0.0580</td>
<td>0.0355</td>
<td>0.1884</td>
<td>N.S.</td>
</tr>
<tr>
<td>12L:12D</td>
<td>♂</td>
<td>C/N ratio</td>
<td>y = 0.0237X + 0.7967</td>
<td>0.1031</td>
<td>0.3211</td>
<td>N.S.</td>
</tr>
<tr>
<td>12L:12D</td>
<td>♀</td>
<td>C/N ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16L:8D</td>
<td>♂</td>
<td>C/N ratio</td>
<td>y = -0.0139X + 1.0633</td>
<td>0.0722</td>
<td>0.2688</td>
<td>N.S.</td>
</tr>
<tr>
<td>16L:8D</td>
<td>♀</td>
<td>C/N ratio</td>
<td>y = 0.0661X + 0.8207</td>
<td>0.2578</td>
<td>0.5077</td>
<td>N.S.</td>
</tr>
<tr>
<td>24L:0D</td>
<td>♂</td>
<td>C/N ratio</td>
<td>y = -0.0139X + 0.9147</td>
<td>0.0779</td>
<td>0.2791</td>
<td>N.S.</td>
</tr>
<tr>
<td>24L:0D</td>
<td>♀</td>
<td>C/N ratio</td>
<td>y = -0.0255X + 1.1173</td>
<td>0.2829</td>
<td>0.5319</td>
<td>S.</td>
</tr>
</tbody>
</table>

R* coefficient of determination  
r** coefficient of correlation  
S, N.S.*** significant or not significant at p = 0.05
<table>
<thead>
<tr>
<th>PHOTOPERIOD</th>
<th>SEX</th>
<th>MEAN C/N RATIO</th>
<th>S.E.</th>
<th>MEAN C/N RATIO (Males plus Females)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0L:24D</td>
<td>♂</td>
<td>.78</td>
<td>.04</td>
<td>.78</td>
</tr>
<tr>
<td>2L:22D</td>
<td>♂</td>
<td>.86</td>
<td>.05</td>
<td></td>
</tr>
<tr>
<td>2L:22D</td>
<td>♀</td>
<td>.85</td>
<td>.06</td>
<td>.86</td>
</tr>
<tr>
<td>4L:20D</td>
<td>♂</td>
<td>.85</td>
<td>.04</td>
<td></td>
</tr>
<tr>
<td>4L:20D</td>
<td>♀</td>
<td>.93</td>
<td>.06</td>
<td>.89</td>
</tr>
<tr>
<td>8L:20D</td>
<td>♂</td>
<td>.95</td>
<td>.03</td>
<td></td>
</tr>
<tr>
<td>8L:20D</td>
<td>♀</td>
<td>.99</td>
<td>.05</td>
<td></td>
</tr>
<tr>
<td>8L:20D</td>
<td>♀</td>
<td>.90</td>
<td>.04</td>
<td>.95</td>
</tr>
<tr>
<td>12L:12D</td>
<td>♂</td>
<td>1.18</td>
<td>.06</td>
<td></td>
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<tr>
<td>12L:12D</td>
<td>♀</td>
<td>.93</td>
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<td>1.06</td>
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<td>16L:8D</td>
<td>♂</td>
<td>.99</td>
<td>.05</td>
<td></td>
</tr>
<tr>
<td>16L:8D</td>
<td>♀</td>
<td>1.18</td>
<td>.12</td>
<td>1.08</td>
</tr>
<tr>
<td>24L:0D</td>
<td>♂</td>
<td>.84</td>
<td>.05</td>
<td></td>
</tr>
<tr>
<td>24L:0D</td>
<td>♀</td>
<td>.90</td>
<td>.05</td>
<td>.87</td>
</tr>
</tbody>
</table>

S.E. - standard error

Figure 35 depicts mean ratio changes for cells from males only, females only and males plus females with the respective photoperiod of the test animal.
Figure 35. Mean C/N ratio changes for cells from males only, females only and from males plus females plotted relative to the respective photoperiod of the test animals. The curve drawn through their mean value shows an upward trend with increasing number of light hours.
2L:22D (0.86) and 4L:20D (0.85). Mean C/N ratio values for females increased from 2L:22D (0.85) to 4L:20D (0.93). At 4 (0.93), 8 (0.95) and 12 (0.93) light hours, ratio values remained fairly stable. A sharp increase occurred at 16L:8D (1.18) which was followed by a sharp decrease at 24L:0D (0.90). The latter value is, however, similar to that of 2L:22D (0.85), 4L:20D (0.93), 8L:16D (0.95) and 12L:12D (0.93). Combined mean ratio values for males plus females showed an upward trend with increasing number of light hours. The highest values occurred at 12L:12D (1.06) and 16L:8D. This value was comparable to that obtained for 2L:22D (0.86) and 4L:20D (0.89). Regression and correlation analyses were performed on cell area versus serial section number for all test animals. The results are tabulated in Table 12.
<table>
<thead>
<tr>
<th>PHOTOPERIOD</th>
<th>SEX</th>
<th>PARAMETER</th>
<th>EQUATION OF LINE ( y = ax + b )</th>
<th>( R^* )</th>
<th>( r^{**} )</th>
<th>Significance ** ***</th>
</tr>
</thead>
<tbody>
<tr>
<td>0L:24D</td>
<td>0</td>
<td>Cell Area</td>
<td>( y = 0.3701X + 25.43 )</td>
<td>0.3011</td>
<td>0.5490</td>
<td>N.S.</td>
</tr>
<tr>
<td>2L:22D</td>
<td>0</td>
<td>Cell Area</td>
<td>( y = -0.2943X + 29.28 )</td>
<td>0.0792</td>
<td>0.2814</td>
<td>N.S.</td>
</tr>
<tr>
<td>2L:22D</td>
<td>0</td>
<td>Cell Area</td>
<td>( y = -0.2872X + 26.28 )</td>
<td>0.2354</td>
<td>0.4852</td>
<td>N.S.</td>
</tr>
<tr>
<td>4L:20D</td>
<td>0</td>
<td>Cell Area</td>
<td>( y = -0.0401X + 23.35 )</td>
<td>0.0053</td>
<td>0.0731</td>
<td>N.S.</td>
</tr>
<tr>
<td>4L:20D</td>
<td>0</td>
<td>Cell Area</td>
<td>( y = -0.6012X + 33.14 )</td>
<td>0.3870</td>
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<td>N.S.</td>
</tr>
<tr>
<td>8L:16D</td>
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<td>Cell Area</td>
<td>( y = -0.5057X + 23.86 )</td>
<td>0.6085</td>
<td>0.7801</td>
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</tr>
<tr>
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<td>Cell Area</td>
<td>( y = -0.0685X + 26.95 )</td>
<td>0.0099</td>
<td>0.0999</td>
<td>N.S.</td>
</tr>
<tr>
<td>8L:16D</td>
<td>0</td>
<td>Cell Area</td>
<td>( y = -0.3181X + 32.47 )</td>
<td>0.2739</td>
<td>0.5233</td>
<td>N.S.</td>
</tr>
<tr>
<td>12L:12D</td>
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<td>Cell Area</td>
<td>( y = -0.0111X + 34.92 )</td>
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<td>0.0460</td>
<td>N.S.</td>
</tr>
<tr>
<td>12L:12D</td>
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<td>Cell Area</td>
<td>( y = -0.1697X + 33.68 )</td>
<td>0.0141</td>
<td>0.1188</td>
<td>N.S.</td>
</tr>
<tr>
<td>16L:8D</td>
<td>0</td>
<td>Cell Area</td>
<td>( y = -0.3028X + 25.56 )</td>
<td>0.4635</td>
<td>0.6808</td>
<td>S.</td>
</tr>
<tr>
<td>16L:8D</td>
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<td>Cell Area</td>
<td>( y = 0.1970X + 26.61 )</td>
<td>0.0394</td>
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<td>N.S.</td>
</tr>
<tr>
<td>24L:0D</td>
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<td>Cell Area</td>
<td>( y = 0.1067X + 29.35 )</td>
<td>0.0280</td>
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<td>N.S.</td>
</tr>
<tr>
<td>24L:0D</td>
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<td>Cell Area</td>
<td>( y = -0.3059X + 31.64 )</td>
<td>0.3159</td>
<td>0.5621</td>
<td>S.</td>
</tr>
</tbody>
</table>

\( R^* \) coefficient of determination
\( r^{**} \) coefficient of correlation
S, N.S.*** significant or not significant at \( p = 0.05 \)
**Ultimobranchial Glands**

The ultimibranchial glands occur as epitheloid tissue surrounding both large and small blood vessels (Figure 36). They are lateral and dorsal to the pharynx and pharangeal teeth, just anterior to the transverse septum and proximal to the sinus venosus. The tissue in which the ultimobranchial glands occur is primarily a haemopoetic tissue mass in which there are also numerous, scattered thyroid follicles. The vessels surrounded by ultimobranchial tissue are thought to be the anterior veins and venules draining into the Common Cardinal vein. Two different portions of glandular tissue can be distinguished in the goldfish ultimobranchial gland; an epithelial portion lining vessels and an "acinar" portion peripheral to the epithelial layer.

The epithelial component borders the basal lamina of the endothelial cells of adjacent vessels; capillaries, venules, possibly venous sinuses and/or lymphatic vessels. It is of variable appearance and includes cells ranging from simple cuboidal through simple columnar to pseudostratified columnar. The combination of epithelium with a very thin endothelium gave the appearance of a lumen to the gland, but a true lumen was not found in any of the goldfish ultimobranchials examined. These epithelial cells were always oriented toward the lumen of the vessel; that is, the apices of these cells
were toward the endothelial layer while nuclei were deeper in the cell and away from it.

The "acinar" portion of the gland which occurred peripheral to much of the epithelial portion consisted of acini of variable size and shape, though they were usually bulbous or flask-shaped. A variety of morphologies were observed; simple, or more rarely compound acini, small or large. All were peripherally invested with connective tissue. The neck regions of these acini, which consisted of the apical portions or processes or product(s) of the acinar cells, extended up into and through the epithelial region and abutted the endothelium. Epithelial portions of the gland surrounding small capillaries were quite similar in appearance to "acini" and could be easily mistaken for "acini".

The gland cells were variable in appearance, exhibiting a continuum of morphologies ranging from large, plump, clear, vacuous cells to small, eosinophilic, low cuboidal cells. For convenience of description, these were divided into four categories: i) cuboidal-eosinophilic, ii) columnar-eosinophilic, iii) plump-clear, iv) deflated-basophilic. The cuboidal-eosinophilic type, which usually lined vessel peripheries and the distal portion of the acini, were characterized by round or oval, central to basal nuclei. Their cytoplasm was usually eosinophilic, but sometimes
displayed a strong basophilic tinge. The columnar-eosinophilic cells varied from a low to relatively elongate form with basal nuclei. In some instances, the typically eosinophilic cytoplasm was relatively basophilic.

Plump-clear cells were normally much larger than the other cell types and ranged in shape from round through flask-shaped to columnar or cuboidal. Their nuclei varied from small and pyknotic to large and vesicular and in position from central to markedly eccentric. The cytoplasm was clear and vesiculated. Normally it displayed a basophilic cast, but occasionally appeared to have an eosinophilic tinge. This cell type usually occurred centrally and apically in the acini. In the epithelium, the bulk of the cells projected below the general epithelium and were connected to the basal lamina proximal to the endothelium by a cytoplasmic projection. Finally, the deflated basophilic cells were usually elongate, with central, markedly oval nuclei and clear cytoplasm of basophilic tinge. They were usually found laterally in the acini, but also occurred frequently in the epithelial portions of the glands.

PHOTOPERIOD EXPERIMENTS

Clear distinctions within the cuboidal-columnar, eosinophilic-basophilic continuum of morphologies exhibited by the cells was not always possible. Accordingly, only
counts of clear versus all other cell types were carried out for each photoperiod. Approximately 1000 cells from combined males and females were counted for each photoperiod.

**TABLE 13.**

<table>
<thead>
<tr>
<th>Photoperiod</th>
<th>Clear</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>0L/24D</td>
<td>30%</td>
<td>70%</td>
</tr>
<tr>
<td>2L/22D</td>
<td>30%</td>
<td>70%</td>
</tr>
<tr>
<td>4L/20D</td>
<td>31%</td>
<td>60%</td>
</tr>
<tr>
<td>8L/16D</td>
<td>30%</td>
<td>70%</td>
</tr>
<tr>
<td>12L/12D</td>
<td>25%</td>
<td>75%</td>
</tr>
<tr>
<td>16L/8D</td>
<td>26%</td>
<td>74%</td>
</tr>
<tr>
<td>24L/0D</td>
<td>20%</td>
<td>72%</td>
</tr>
</tbody>
</table>

Despite the apparent uniformity of distribution of (Table 13) "clear" versus "other" cell types at the various photoperiods, examination of glands from the different photoperiod groups revealed consistent variations in appearance and these are described below.

**Histological Distinctions in the Ultimobranchial Gland With Photoperiod.**

**0L:24D** (Figure 37) - The epithelium around the vessels was composed almost exclusively of eosinophilic columnar cells with oval and basal nuclei. The acini were round with their
Figure 36. Ultimobranchial tissue surrounding a blood vessel (BV) with adjacent haemopoetic tissue (H) and diffuse thyroid (pointer). Mag. X 70.

Figure 37. OL:24D. Characterized by eosinophilic columnar cells (T) adjacent to the lumen (L) and acini (A) composed of cuboidal to low cuboidal (c) basal cells and plump, clear central cells. Mag. X 1500.
distal portions consisting of cuboidal to low cuboidal eosinophilic cells with apical nuclei. These cells formed what appeared to be a complete, slightly scalloped, single layer of cells around the periphery of the gland. Very few recognizable neck regions of acini were noted to extend through the epithelium to the basal lamina of the endothelium. The clear cells, which in this photoperiod occurred only in the centres of the acini, appeared plump and very clear and gave the cells an empty appearance. Clear cell nuclei were from large, oval and somewhat vesicular and pyknotic, or were pushed to the side of the cell membrane as in fat cells which are full of product.

2L:22D (Figure 38) - The tissues from animals acclimated to this regime were similar in appearance to those of the 0L:24D group.

4L:20D (Figure 39) - Vessel linings were composed of roughly equal proportions of cuboidal and columnar cells. Some of the cells (cuboidal) were eosinophilic while the others possessed basophilic cytoplasm. Nuclei were basal in position. The acini were well defined with cuboidal to high cuboidal, eosinophilic, peripheral and basal cells. Acini varied in form from round to flask-shaped and most exhibited prominent neck regions extending to vessel surface epithelium. Their central clear cells were large and plump with flocculated
Figure 38. 2L:22D. Characterized by eosinophilic columnar cells (T) adjacent to the lumen (L) and acini (A) composed of cuboidal to low cuboidal (C) basal cells and plump, clear central cells. The apparent difference in appearance between this section and Figure (above) is due to a common artifact of fixation that occurred randomly in sections of fish at these two photoperiods. Mag. X 1500.

Figure 39. 4L:20D. Characterized by an epithelium of cuboidal (C) and columnar (T) cells adjacent to the endothelial cell (pointers) lining of the lumen (L). Acini (A) are of variable shape and composition. Mag. X 1500.
cytoplasm and relatively large round to oval nuclei.

8L:16D (Figure 40) - The vessel epithelium had an irregular appearance because the mainly columnar cells possessing basophilic cytoplasm were interspersed with flask-shaped, clear cells whose necks expanded beneath the general level of the epithelium. Acini were well defined with large cuboidal to high cuboidal basal and lateral cells. Some were round, distended and "full" in appearance. These usually contained large, plump, flocculated clear cells. Others were more fusiform in shape and appeared to be partially collapsed and emptier. These usually contained fusiform-shaped clear cells.

12L:12D (Figure 41) - The vessel epithelium was similar to that noted in the previous photoperiod. Acini were variable in shape and contained fewer, large, round, distended clear cells and more fusiform cells than was the case with the 8L:16D animals.

16L:8D (Figure 42) - In these specimens, the vessel epithelium was discrete and continuous and composed mainly of basophilic and eosinophilic columnar cells. There were fewer clear cells hanging below the epithelium than in the two previous photoperiod groups and they appeared smaller, more elongate and "grey". Acini generally contained few clear cells and sometimes only a single cell. Many of these
Figure 40. 8L:16D. Characterized by an epithelium of columnar cells (T) and flask-shaped clear cells (F) expanded beneath the base of the general epithelium. Acini (A) are generally well defined with large cuboidal, basal and lateral cells (c). Mag. X 1500.

Figure 41. 12L:12D. Similar to the previous photoperiod with numerous acini (A) of variable shape. Mag. X 1500.
Figure 42. 16L:8D. Characterized by an epithelium of basophilic and eosinophilic columnar cells (T), variable acini often with "deflated" cells (pointers) and basal eosinophilic, large, cuboidal cells (c). Mag. X 1500.

Figure 43. 24L:0D. Large eosinophilic columnar cells (T) line the vessel lumen (L) and acini (A) are similar to those in the 0L:24D photoperiod with plump, clear cells, some with pyknotic nuclei (pointer) and cuboidal eosinophilic basal cells (c). Mag. X 1500.
clear cells appeared elongate and deflated with "grey" cytoplasm. Basal acinar cells were usually eosinophilic and of a large cuboidal form.

24L:0D (Figure 43) - Except for the eosinophilic columnar vessel epithelium, these glands appeared similar to those noted in the 0L:24D and 2L:22D photoperiods.
DISCUSSION

Pituitary-Prolactin Cells of the Rostral Pars Distalis

In all teleosts, adenohypophyseal morphology is basically similar in that the pars distalis is divided into two zones - rostral and proximal (Leatherland, 1975). The rostral pars distalis shows some interspecific variation. In eels, *Anguilla anguilla*, *Anguilla* sp. and salmonids, *Oncorhynchus kisutch*, *O. nerka* and *Salmo gairdneri*, there is a primitive follicular arrangement of prolactin cells, whereas in more advanced teleosts, such as *C. auratus*, the rostral pars distalis is composed of a large mass of prolactin cells interspersed with chromophobes and occasionally basophils (Leatherland, 1975). Bell (1938) characterized the pars anterior of the goldfish as having a perforated appearance in transverse section while in sagittal section, the cells appeared to be divided into horizontal cords several cells in depth. Initial examination of prolactin cells in *C. auratus* gave the impression that they were arranged in cords and clumps; however, in reality they are a continuous mass (Leatherland, 1975) forming the bulk of the RPD. It is the vessels, sinuses, connective tissue and chromophobes within the matrix of the prolactin cells that produce the superficial discontinuity. Leatherland (1975) stated that the prolactin cells of the goldfish were "without apparent orientation"
within the gland" and suggested that there was evidence that cells were "polarized into secretory and synthetic cell areas". Schreibman, et al., (1973) suggested that caution be exercised when drawing conclusions from the morphology of these cells, for although a morphological uniformity is often implied in the description of prolactin cells, the RPD cells "differ in their dimensions, shape of nuclei and degree of granulation and time in which they respond to physiological challenge". He further stated that frequently, prolactin cells possessing similar morphological characteristics are zonated and apparently, this zonation varies among species. For instance, arrangement of these cells in *Xiphophorus* is such that those in peripheral areas have fewer granules and are smaller than the larger cells which are more centrally located and are closest to blood vessels. Conversely, in *G. aculeatus*, prolactin cells in the vicinity of capillaries in the periphery of the RPD exhibit morphological characteristics indicative of a higher secretory activity than those more centrally located. These observations suggest differences in the cells related to synthetic and secretory activity. In the present study, two tissue columns were examined. Although both included what could be considered "peripheral regions", one of the columns was punctuated by vascularization while the other was from an area of little vascularization and was termed avascular. Changes in the morphology of the cells comprising each tissue core were evident from the variation in C/N ratio.
These changes in both cores resulted in ratio based wave patterns of relatively consistent and approximately corresponding periodicity (Figure 12 a & b). Amplitudes, however, differed. Those cells from the core located in the more vascularized region had an amplitude one half that of the tissue-core-cells located away from blood sinuses. There is a fairly even distribution of data points along the curved trend line in Figure 12 a and approximately 25% of all points within this data set can be assigned to either peak, trough, upslope or downslope regions of the wave. This allotment of data points is not as clear-cut in the corresponding graph (Figure 12 b). In some cases (wave 2 and 3) C/N ratio values increase rapidly from low upslope values to peak values, but a more even distribution of the points along this curve occurs in wavelength 1 and 4. It is suggested that the four distinguishable phases comprising the wave are the result of morphological changes indicative of a four phase secretory cycle. Though fully cognizant of the pitfalls inherent in synthesizing a dynamic secretory cycle from data obtained from what is of necessity a temporally static specimen, the sinuous continuity of the wave patterns generated from the section-by-section analysis of the gland virtually necessitates a dynamic interpretation.

Histological support for a cyclical secretory pattern was acquired through examination of the cells comprising each
of the 4 phases of the C/N ratio produced waves. The majority of cells that correspond to "peak" regions (high C/N ratio values) possess an abundant cytoplasm believed to contain a large amount of secretory product. The nucleus is plump and nucleoli are absent. It is thought that these cells are in a storage state or may already be slowly releasing product. Alternatively, it is possible that these cells may be involved in the concurrent production and release of the hormone prolactin. The majority of cells that correspond to trough regions appear to have small amounts of cytoplasm and large, round to oval nuclei with prominent nucleoli. This morphology is suggestive of cells in preparation for active synthesis of a proteinaceous product, primarily through ribosome production or in the early stages of active product synthesis. The majority of cells in upslope regions (increasing C/N ratio values) demonstrate an increase in amount of cytoplasm over those cells in trough regions. Also, cells in higher upslope regions have more cytoplasm than those cells located lower on the slope. Nuclei are round to oval in shape. Nucleoli in these cells become less evident in high upslope regions. It is thought that these cells are in the process of actively synthesizing secretory product. Cells corresponding to downslope regions demonstrate a progressive decrease in the amount of cytoplasm with location on the downslope. Higher up in the downslope region, nuclei
of these cells are round to oval, but lower down on the slope, progressive irregularity of outline and folding occurs and results in kidney-shaped nuclei. Infrequently, two small nuclei per cell may be encountered. Nucleoli become increasingly obvious with the progression down the slope. Overall cell morphology suggests that these cells are probably involved in the progressive release and final stages of product release. The occurrence of cells in lower downslope regions, with small rounded nuclei (sometimes two per cell) and clumped chromatin is not inconsistent with this view, since such characteristics are common in relatively inactive cells.

Schreibman, et al. (1973) state that cytological profiles of glandular elements change in relation to the demands placed on them and that standard parameters used to assess cytological activity in prolactin cells are: extent of granulation, cell and nuclear dimensions, prominence of nucleoli, cytoplasmic RNA, ER, number and/or density of cristae, number of Golgi bodies and indications of granule release. Changes in C/N ratio has been used in this study as an overall indicator of changes in cell activity.

The relation between cytoplasmic and nuclear area changes coupled with variations in nuclear shape and the appearance or absence of nucleoli, enable a direction to be attributed to the C/N ratio-produced wave pattern. Average cytoplasmic
areas exhibited a cyclic change which ranged from $16 \pm 7 \, \mu m^2$ in peaks to $12 \pm 4 \, \mu m^2$ in troughs and $13 \pm 5 \, \mu m^2$ in both upslope and downslope regions of the tissue core from the avascular region. A similar cyclic size (area) change was observed in the corresponding core from the more vascular region. Peak cytoplasmic areas of $16 \pm 2 \, \mu m^2$ fell to $11 \pm 4 \, \mu m^2$ in trough regions and upslopes and downslopes had intermediate values of $11 \pm 3 \, \mu m^2$ and $12 \pm 4 \, \mu m^2$ respectively. These changes lend credence to the probable cyclical manufacture and release of product, but by themselves do not suggest the direction of the cycle. However, when changes in cytoplasmic area are coupled to changes in nuclear area and appearance, as well as to the presence or absence of nucleoli in these nuclei, strong indicators of cyclic direction result.

Leatherland and Ensor (1973; in Holtzman and Schreibman, 1975) employed nuclear measurements as indicators of prolactin cell activity in \textit{C. auratus}. Holtzman and Schreibman (1975) state that "synthesis must be distinguished from secretory (release) activity" and that it is the combination of "large nuclear size, prominent nucleoli, perinuclear halos and the abundance of intensely stained secretory granules that reflect synthetic activity in the prolactin cells of \textit{Xiphophorus maculatus} in freshwater". In \textit{C. auratus}, trough cells possess large round nuclei ($21 \pm 7 \, \mu m^2$ and $19 \pm 7 \, \mu m^2$ - avascular and vascular regions respectively) with prominent...
nucleoli and euchromatin. They also have scant amounts of cytoplasm of basophilic cast that is probably indicative of ribosomes on the rough endoplasmic reticulum. This combination of characteristics is usually indicative of both transcriptional and translational activity by cells that synthesize a proteinaceous secretory product. In peak regions, the round to oval-shaped nuclei are smaller than those of trough cells: \( 16 \pm 7 \, \mu m^2 \) vs \( 21 \pm \mu m^2 \), avascular core; \( 16 \pm 2 \mu m^2 \) vs \( 19 \pm 7 \mu m^2 \), vascular core. Generally nucleoli are absent and some heterochromatin is obvious in the nucleus. Cytoplasmic areas are greatest in the peak region. Relative to trough cells, these characteristics indicate a decrease in nuclear activity (smaller size, lack of nucleolus) and therefore a decrease in the production of mRNA. The large amounts of cytoplasm in these cells suggest apparent activity in the manufacture of secretory product but, with no new synthesis of mRNA, protein synthesis must cease or slow down with completion of protein synthesis on the ribosomes of the RER or on ribosomes free in the cytoplasm. This morphology of small nuclei, lack of nucleoli and initial appearance of heterochromatin, along with the euchromatin and abundance of secretory product, suggests that the cells are in a storage state or in the latter stages of protein synthesis and may have begun to release their product. They may be involved in concurrent release and synthesis of
product.

Cells in upslope regions have round to oval nuclei which are intermediate in size between trough and peak values: $21 \pm 7 \, \mu m^2$ (trough) vs $19 \pm 5 \, \mu m^2$ (upslope) vs $16 \pm 7 \, \mu m^2$ (peak), avascular region; $19 \pm 7 \, \mu m^2$ (trough) vs $18 \pm 4 \, \mu m^2$ (upslope) vs $16 \pm 2 \, \mu m^2$ (peak), vascularized region. Nucleoli became less evident in upslope regions. Euchromatin is obvious. Cytoplasm in these cells shows an increase in area over that of trough cells. This profile suggests that transcription is still taking place but, the concomittant increase in cytoplasm, coupled to the disappearance of nucleoli suggest that translation of the mRNA on the ribosoms and subsequent synthesis of the secretory product is the principal activity in which the cell is engaged at this time. Whatever the case, it is definitely the profile of a cell engaged in active synthesis of a proteinaceous product.

Cells in downslope areas have nuclei which are round, oval and kidney-shaped. They are slightly smaller than those in nuclear areas in upslope regions: $16 \pm 6 \, \mu m^2$ vs $19 \pm 5 \, \mu m^2$, avascular region; $17 \pm 3 \, \mu m^2$ vs $18 \pm 4 \, \mu m^2$, vascular region. More nucleoli are present in cells in lower downslope regions than in cells higher up on the slope. Heterochromatin is noted more frequently in cells in high downslope regions than in those closer to trough regions where euchromatin is usually found in the nuclei. The area of cytoplasm ($13 \pm 5 \, \mu m^2$
avascular region; $12 \pm 4 \mu m^2$ vascular region) is less than in peak cells ($16 \pm 7 \mu m^2$, avascular region; $16 \pm 2 \mu m^2$, vascular region). Cells high on the downslope have noticeably more cytoplasm than is found in cells lower on the downslope. This profile suggests that the cell seems to be synthetically inactive in high downslope regions and is probably involved in the release of product. However, lower down on the slope, these cells show evidence of reorganization of the cellular "machinery" for synthesis in the presence of nucleoli and euchromatin in the plump nuclei. Infrequently, cells with two small nuclei were encountered in lower downslope regions and it is suggested that cell division, if it is going to take place, could logically do so here. Therefore, the cycle has a left to right direction which accordingly corresponds to left and right on the graphs. In the animal, however, the cycle is in a dorsal to ventral direction.

In summary, the recognition of peak and trough regions is more easily achieved than the discrimination of upslope from downslope regions. In the latter cases, all subtle changes in morphology, e.g. nuclear shape and number per cell and the presence or absence of nucleoli, coupled with changes in cytoplasm, must be closely scrutinized in a serial section-by-section examination. Recognition of these morphological differences then allows cells to be allotted to the proper regions and consequently, the direction of the cell cycle can
be determined.

The Wave Pattern

A sample population of approximately 5,000 cells is represented by the C/N ratio produced waves of each tissue core. Based on the data generated by Quantimet analysis and exhaustive histological examination, it is not unreasonable to suggest that individual prolactin cells appear to undergo a form of cyclical activity which is broadly divisible into four phases: i) synthesis, ii) storage, iii) secretion and iv) reorganization and preparation for synthesis. Linear regression analysis for each of cytoplasmic area (Figures 6 and 10 and Table 2), nuclear area (Figures 5 and 9 and Table 2), cell area (Figures 7 and 11 and Table 2) and C/N ratio (Figures 8a and b and Table 2) resulted in lines whose slopes approached zero and whose correlation coefficients were not significant. Examination of all the points comprizing each data set for the various parameters examined suggested that their distribution showed "groups" or "clusters" of points above and below their respective least squares line-of-best-fit and that the most frequently observed distribution was suggestive of a wave-like pattern. This indicated the possibility of some cyclical morphological variation along the tissue cores.

Nuclear Area - The distribution of the values for the nuclear
area data set from the vascular region did not show any readily discernible pattern in fluctuation whereas those for the avascular region produced a very irregular two wave pattern. Neither set of data could be used in any consistent manner to relate to histological change (Figures 5 and 9).

Cytoplasmic Area - The distribution of the points for the data set for the vascularized region weakly suggested an irregular wave-like pattern of two to three rather irregular waves that did not fit well with the C/N ratio-generated waves. Those for the avascular region strongly suggested a four wave pattern and although they did not fit precisely with the C/N ratio generated wave pattern, they approximated it. These data were, however, not very useful for indicating changes taking place in the cytoplasm of the cells. This was surprising, especially in the latter case, because it seemed that cytoplasmic variation could be predicted from the C/N ratio plots. In some instances, especially in the case of the cytoplasmic areas in the avascular region, high and low areas did not correspond with comparable regions from the C/N ratio graphs and therefore also with histological appearances. The cytoplasmic area plot for cells from the vascular area, although suggestive of some correspondence, in fact corresponded poorly with the comparable regions of the C/N ratio graph and was therefore a poor predictor of cytoplasmic changes, (Figures 6 and 10).
A number of reasons could account for this approximate correspondence. Area or volume measurements are subject to considerable variability due to "vagaries" of technique. For example, work done on the Corpuscles of Stannius in the present study indicated that a 30% difference in average area had been induced between serial sections located on two adjacent slides. This was found to be related to differential flattening of the material during processing. Therefore, it is possible that, in the case of the pituitary analyzed from the various slides, there could easily have been differential flattening between the material on the two slides as adjacent rows of sections and even of adjacent sections themselves. This would certainly tend to mask, or at least disrupt the pattern of the size fluctuations found in cytoplasmic area measurement. However, the use of C/N ratio, given that nucleus and cytoplasm are affected similarly by technique, would eliminate any pattern disruption caused by the swelling, shrinking or stretching of the tissue during manipulation. A substantial portion of the problems of correspondence and predictability may be accounted for by non-uniformity of technique. It is also quite possible that there are populations of cells of different sizes. This was noted by Schreibman (1964), and Holtzman and Schreibman (1972; cited in Schreibman, et al., 1973) for Xiphophorus maculatus in which the peripheral prolactin cells appear smaller and less
granular compared to the larger, more granulated, centrally-located prolactin cells which in this fish are closest to the blood vessels. Conversely, in *Gasterosteus aculeatus*, peripheral prolactin cells show morphological characteristics which are indicative of higher secretory activity than those in central locations (Leatherland, 1970 a; cited in Schreibman, et al., 1973). Emmart (1969) noticed that cells in the peripheral area of the rostral pars distalis of *C. auratus* possessed fewer granules than cells in deeper locations. These observations were interpreted as areas with different synthetic and secretory capabilities. From Figure 6 of the avascular region, the peak in cytoplasmic area between section numbers 30 to 40 is much higher than that of any other peak and would tend to support the theory that there may be areas in which the cells are larger. This type of difference in cell populations would very definitely disrupt the smoothness of any wave pattern based on area analysis and, would again produce problems with predictability and the correspondence of the area analysis with the C/N ratio analysis. Why groups of cells in a supposedly uniform gland should be of different basic sizes is, of course, not known. However, the use of C/N ratio should negate much of the pattern disruption caused by size differences among populations of cells, assuming, once again, that relative cytoplasmic to nuclear ratios remain stable.
within the size change range.

Despite the foregoing, it is the large fluctuations in cytoplasmic area that are largely responsible for the wave pattern produced in the C/N ratio plot (Figure 12a,b). It appears that some of the fluctuation in nuclear area (Figure 5), has somehow regularized the pattern apparent in C/N ratio plot (Figure 12a) making it much more evident or stable than the similar pattern expressed by the avascular region cytoplasmic area (Figure 6) plot alone. Similarly, the fluctuations in nuclear area have rendered the pattern for the C/N ratio plot (Figure 12b) for the vascular region tissue core more evident than that shown by the vascular cytoplasmic area plot (Figure 10) or the nuclear area plot (Figure 9) alone.

**Cell Area** - The plot for total cell area (Figure 11) of the vascular region shows a distribution of data points in the form of two low amplitude waves. That for the avascular region (Figure 7) suggested two irregular wavelengths of high amplitude. These plots do not correspond well with any of the graphs previously described and could not be used to interpret cyclical changes associated with the secretory cycle within the gland. As previously stated, the use of area as an indicator of cellular activity has pitfalls and drawbacks. It is possible that in these core analyses, different areas were composed of cells of different sizes.
(Schreibman, et al., 1973), that the two tissue columns represent different cell populations and that subsequent comparison of their areas, in the context of the present work, as a measurement of cell activity, is probably meaningless. When total area measurements were compared with selected histologically-verified C/N ratio peaks and troughs, some interesting relationships or lacks thereof, appeared. For instance, in the first trough (section number 5) of the avascular plot (Figure 7) the total average area of the approximately 150 trough cells is 40 µm² which is a very large area value, yet the cells have a low (trough) C/N ratio value. On the other hand, in section number 31, a low total average area for the approximately 150 measured cells of 23.5 µm² corresponded with a low (trough) C/N ratio value. And, to further complicate matters, section number 16, with a median total average area for the approximately 150 measured cells of 32.5 µm² also corresponded with a low (trough) C/N ratio value. Similar randomness of relationships could be demonstrated for peak areas. The interesting phenomenon demonstrated here is that histologically and in reference to C/N ratio, trough cells with little cytoplasm but active nuclei can vary in size by a factor of approximately two; that is, 23.5 µm² versus 40 µm² in total area. This emphasizes the danger of using area, volume or diameter measurements as indicators of activity level of
prolactin cells, especially in small samples. Holtzman and Schreibman (1975) state that although cytometry is an accepted method employed in the analysis of pituitary cytology, it has its shortcomings. Generally, they feel that the measurement of cell indices may introduce bias as there may be the tendency to select those cells which are larger and more intensively stained for measurement. Nuclear indices may not always be reliable either and the technical difficulties in measuring small (3-4 μm in diameter) nuclei can be appreciated. They suggest that cell density (C.D. = number of cells per 303 μm²) determination probably produces more acceptable results because a larger cell population is sampled. In the present study, on the initial gland analyzed, the number of cells occupying an area of 183 μm or 610,000 μm² were analyzed and, it is based on the findings therein, that caution is suggested concerning the application of previously-accepted cytometrical methods and the interpretation of the data generated thereby. These methods have been used in studies by many researchers, among them Olivereau and Ball (1966), Olivereau (1968b), Ball and Ingleton (1973), Leatherland, et al., (1974), Nagahama, et al., (1975), Ethridge and Benjamin (1977), Benjamin (1979) and Benjamin and Williams (1979). Schreibman, et al., (1973) reviewed a great deal of literature in which these methods had been employed and also discussed problems pertaining to
the evaluation of cell activity, by methods presently available. In many cases, the test animals were placed in experimental conditions that produced enough stress on the prolactin system to disrupt its normal functions and to produce measurable differences in volume, area or nuclear diameter that correlated with experimental conditions. Exactly what those measurable differences might have indicated regarding secretory activity level is, in the light of the present findings, an open question.

Since the two high amplitude wave patterns produced by average cell areas from the avascular region (Figure 7) appear to bear little relation to secretory cycle, it can be tentatively concluded, from the non-randomness of the curve, that populations of basically different cell sizes were sectioned in the path of the vertical tissue column through the gland. The reason for this is inexplicable at present. The low amplitude wave pattern generated from the tissue core from the vascular region could be interpreted to indicate that the cells were of basically uniform size throughout that tissue column. C/N ratio was the only parameter employed which allowed meaningful comparison of the two tissue cores relative to the secretory cycle. Histological verification of the activity states suggested by the position of the cells along the C/N ratio-produced wavelengths adds further credence to the validity of a model for the cytoarchitecture
of this gland. Previously, the pituitary of *C. auratus* has been described as a compact mass (Emmart, 1969). However, the cellular homogeneity implied by this description is misleading. Evidence has been proffered that the pituitary gland of *C. auratus* is composed of a heterogeneous population of cells and that these cells are localized as to their secretory activity. This results in a lamellar-type cellular arrangement within the mass of prolactin cells (Figure 44).

Finally, this measurement (ratio) is ideal for use in studies requiring various cell measurements because the use of ratio minimizes any errors introduced by technique and allows the comparison of different populations of cells from the same or adjacent tissue sections and also between sections contained on different slides. In the present study the use of this parameter therefore enabled the comparative analysis of quantitative data generated for tissue from test animals acclimated to the various photoperiods.

The seeming lack of a linear relationship depicting cellular activity is incompatible with the view of either complete homogeneity, or random heterogeneity of these hormone-producing cells. It is, on the other hand, not incompatible with some form of synchrony of cellular activity that is reflected by a spatial variation (i.e. period) indicative of some regulated cycle of cellular activity. C/N ratio best expresses the latter
and this, coupled with exhaustive histological verification of cells in apparently different states of a secretory cycle which contribute to the C/N ratio produced wavelengths, gives rise to the cytoarchitectural model of a lamellar arrangement of prolactin cells within the rostral pars distalis of C. auratus.

Transects including the cells between the original tissue cores lend support to this model. However, a number of factors rendered this analysis somewhat difficult and inexact: (i) original areas analyzed could not be precisely re-located since co-ordinates had not been recorded for them, (ii) often transects extended peripheral to the area(s) originally analyzed or stopped somewhat short of totally including the vascularized area originally measured, (iii) the initial transect co-ordinate might include only part of the original field analyzed although transect and initial co-ordinates were aligned as closely as possible, (iv) although care was taken to ensure that successive machine fields lay on a transect between the two original co-ordinates, there was the possibility of mechanical errors including the overlapping of these fields or faulty alignment of fields along the transect. All transects extended from the avascular tissue core to the vascularized tissue core. The C/N ratios of the cells analyzed along the transects appears in Table 14 and Figure 17. Transect five (Figure
### TABLE 14.

C/N Ratios Generated for Transects Between the Originally Analyzed Tissue Cores

<table>
<thead>
<tr>
<th>TRANSECT FIELD NO.</th>
<th>TRANSECT NUMBER</th>
<th>(Serial Section Number)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1(3)</td>
<td>1(10)</td>
</tr>
<tr>
<td>1</td>
<td>0.58</td>
<td>0.85</td>
</tr>
<tr>
<td>2</td>
<td>0.87</td>
<td>1.01</td>
</tr>
<tr>
<td>3</td>
<td>0.88</td>
<td>0.77</td>
</tr>
<tr>
<td>4</td>
<td>1.07</td>
<td>0.71</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>1.25</td>
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<tr>
<td></td>
<td>3(12)</td>
<td>4(19)</td>
</tr>
<tr>
<td></td>
<td>0.88</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>1.07</td>
<td>1.07</td>
</tr>
<tr>
<td></td>
<td>0.86</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>0.83</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>0.93</td>
<td>0.85</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.32</td>
</tr>
</tbody>
</table>

17) provided one of the best fits. It extended from a mid-upslope region of a curve in the avascular core to a peak region in the vascular core. The initial transect C/N ratio value of 0.93 coincided closely with the original core area analysis C/N ratio value of 0.96. The terminal transect value coincided precisely with the initial vascular core peak value of 1.03. The two intermediate transect values of 0.85 and 0.87, which are values indicative of mid- to high-upslope positions on avascular and vascular waves respectively, strongly indicate that the transect remained on, and probably climbed an upslope toward the peak of the vascular wave. This transect closely fitted the model which postulates a laminar arrangement of cells relative to secretory cycle
phase. Transect number three also provided good fit for the model. It was run from an avascular peak to a vascular peak. Generally, all of the values derived from the transect were in the peak C/N ratio range which of itself indicated that the transect remained on or close to a peak and thus also demonstrates the lamellar cell arrangement. This transect is an exceptionally long one (5 fields) and did extend peripheral to both of the original areas analyzed. The initial transect value of 0.88 is lower than the value of the initially measured tissue (1.17), but this may be because the first transect measurement was taken from a peripheral region that may have included considerable vascularization. The second reading from the transect is closer to the expected reading from the avascular peak, but is still low. The final transect value (1.25) is somewhat higher than the originally-obtained value of 0.81 and, though high for vascular core peaks, is undoubtedly a peak. These types of very high reading for peaks and very low readings for troughs were not uncommon in the general analysis and they usually occurred in the proper directions; that is higher than "normal" peak readings and lower than "normal" trough readings.

Transect number 2 (Figure 17, Table 14) was chosen for the coincidence of the high shoulder of an avascular upslope region with the high upslope region of the lower amplitude
vascular wave. The expected trend of the transect values would be from relatively high to slightly lower, if the lamellar model were to hold. The general trend fits the model. The initial transect model of 0.85 was approximately the same as the value of 0.82 from the original core. The second reading of 1.01 was very close to that to be expected from the high upslope of an avascular wave. The third transect reading of 0.77 coincided with the expected reading of 0.86 for the high upslope region of a vascular wave. The fourth transect value of 0.71 was somewhat low for a high upslope region, but extended the values in the expected downward direction when the degree of tilt from perfectly frontal was taken into consideration. From serial reconstruction of the pituitary gland, it was determined that the gland had been sectioned with a lateral tilt of about five degrees and an antero-posterior tilt of 10 to 15 degrees away from squarely frontal. Since the bands of synchronous cells were found to be only one or two cells in thickness, or about 9 μm and since the antero-posterior distance over which the transect extended was only about 80 to 100 μm, it was felt that this 10 to 15 degree tilt had little effect on the wave pattern. Since most transects transversed a lateral distance between 250 and 300 μm, it was thought that the five degree tilt from the horizontal would take a transect that began in one phase of the cycle into the adjacent phase, or even through it and
into the second phase removed from it. This would be especially noticeable in transects that began and ended peripherally to the originally analyzed areas. As it is, the wave pattern from the vascular core in Figure 12b is slightly displaced to the left of the avascular wave pattern so that generally its peaks correspond more closely with the upslopes of the avascular core. This effect is thought to be a result of the five degree tilt from horizontal that occurred during the embedding process. Therefore, the 0.71 value of the last transect point could be expected to be lower than the predicted value if, as is suspected, it extended peripherally to the originally-analyzed area from the vascular core.

Transect 4 which was expected to demonstrate a gradual trend from avascular low upslope or trough values of about 0.5 upward to mid-upslope values in the vascular core of about 0.7, did not do so. The trend was gradual, but in the wrong direction, with initial and final transect values (0.83 and 0.32) far different from original values of 0.48 and 0.78. Peripheral extension of the transects and the five degree tilt could be called upon to account for some of the difference, and would extend both ends of the transects in the direction of the shift that is so evident and exaggerated. The second transect point does not coincide with the values obtained from the original analysis and the third transect
point, which roughly coincides with the original value from the vascular core, is the only value that comes close. It is felt that this transect was one in which the original avascular core area was missed entirely in the placement of the first transect point, and that the transect taken was run at a substantial angle to the straight line which would have joined the original two areas analyzed and probably crossed it with the third transect point. In other words, given the lamellar structure, the 15° antero-posterior tilt and the five degree lateral tilt; if the transect started a substantial distance anterior (or posterior) to the initial avascular area analyzed and ran toward the region of the initially analyzed vascular area, the transect values could start substantially higher than that predicted. They would then approach that expected from the vascular area. Finally, if the transect were extended peripherally but posterior to the theoretically correct transect line, values could plummet far more precipitously than initially predicted. Since the transects were done months after the initial core analyses, a substantial deviation of the actual transect line from the intended transect is a distinct possibility and is one of the only plausible explanations that retains the probity of the lamellar model.

A somewhat similar explanation is required for transect number 1 which was supposed to run from an avascular trough to
a trough in the vascular core and should theoretically have shown uniformly low C/N ratio values. Instead, the initial transect point coincided well at 0.58 with the expected trough values, the next two rose progressively through 0.87 and 0.88 to either high downslope or high upslope values and the final point terminated at the 1.07 value of a vascular peak. Although the last three values obtained from the transect deviated in the proper direction, (up a downslope to a peak), when section tilt and transect angle relative to the ideal transect were considered, the deviation was thought to be too large to be accounted for by these factors alone. Excluding machine and operator error, one other plausible explanation is that a combination of transect deviation in addition to a tight warp in the tissue that could have been caused by handling during dissection or tissue processing was responsible for the unexpected readings. This area, near the beginning of the cores, was close to the surface of the gland where the tissue would be prone to damage and warping by instruments during handling.

Transect 6 was run from the high shoulder of an avascular downslope into a not very well defined trough in the vascular core. Expected C/N values would have been expected to show a decline. They remained essentially constant along the transect which was the shortest of the transects. Only three areas were analyzed because the RPD
was narrower at section 40 near the bottom of the gland. Four transects would have extended beyond the edges of the tissue and three were not enough to totally include the initially measured cores. The compromise first transect point registered 0.69 on the C/N ratio scale as opposed to the expected 1.12. This is thought to be a result of measuring central to the avascular core area which one would expect to lower the transect C/N ratio and also of some anterior transposition of the first transect measurement which could easily have lowered the value because of tilt. The initial low downslope to trough value thus obtained would then be expected to hold over the remainder of the transect into the trough of the vascular core, if the lamellar secretory cycle theory were valid. It did, with the last transect point value falling in the mid-range of the points that formed the rather poorly defined vascular region trough.

The foregoing interpretations of the results obtained from the transects demonstrate that there must be enough morphological similarity based on C/N ratio similarity in cells positioned (arranged) across the middle of the RPD of this frontally sectioned gland to postulate a banding pattern. Morphological variation in cells usually is of functional significance and, in this case, the morphological variants can be associated with phases of a prolactin secretory cycle. Therefore, the conclusion that the
prolactin cells of the RPD of the goldfish are arranged in synchronous or synchronized horizontal layers about one cell thick and that phase of secretory cycle is responsible for the morphological characteristics which permit recognition of the layers rather presents itself.

Analysis of C/N Ratio Plots from other Photoperiods

When C/N ratios of prolactin cells were plotted against serial section number for test animals held at the other photoperiods, the trend lines drawn through the data set peculiar to each experimental regime resulted in wave-like patterns which were similar to those generated by the cells from the initially analyzed tissue cores.

Using the model proposed for the cytoarchitecture of the prolactin cells of the rostral pars distalis, the wave patterns derived from the photoperiods could be explained.

The C/N ratio versus section number plot of prolactin cells from an animal maintained at 24L:0D photoperiod (Figure 18) generated a wave pattern of \( \sim 30 \, \mu \text{m} \) period and \( \sim 0.20 \) amplitude through a \( 72 \, \mu \text{m} \) core of tissue. Though the wave form is similar to that of the extensively analyzed 8L:16D gland, hereafter referred to as the control gland, the period and amplitude of the test prolactin area are substantially different from the \( 36 \, \mu \text{m} \) period and 0.40 amplitude of the tissue core from the vascularized region of
the control. The period difference can be explained on the basis of the core having been taken through a population of "small cells. Average cell size in the control gland approximated 9 µm and produced the relatively stable period of 36 µm through both cores. There were in the control gland regions where the wave periods were less than 36 µm and where cell diameters were smaller. In this test gland, the average cell diameter was ~7 µm. A four phase synchronous secretory cycle arranged in a laminar fashion with cells of 7 µm diameter, would produce a period of about 30 µm. As was noted from the total area plot in the control gland, quite large total area differences (from 20 to 40 µm²) may occur in populations of prolactin cells. Though a 100% area difference would not be translated into such a large diameter difference in the roughly spherical cells, the smaller cells would result in cells at the lower end of the 5 to 10 µm diameter range scale for prolactin cells in the goldfish.

With the postulated laminar arrangement of cells relating to a secretory cycle, there are two plausible explanations for the occurrence of an amplitude considerably lower than that normally expected and similar to that noted for this test animal. One is a mechanical explanation; the other is based on function and is, therefore, conjectural because there is no direct evidence for it.

If the plane of section is such that it parallels the
layers of cells the amplitude of the wave pattern generated by the phasic C/N ratio changes will be maximal. If the section plane is tilted so that it passes through two cell layers only, given that the postulated four phases of the secretory cycle are arranged in one cell thick laminar bands responsible for the sine-like wave, the amplitude will be truncated, because low trough values and high peak values cannot be obtained. Sections crossing two cell layers would always include either upslopes or downslopes, with troughs and peaks and thereby lower the C/N values. With the section plane tilted to cross through three cell layers only, further truncation of amplitude would occur, because the highest value recordable could include only one third peak values plus two thirds upslope/downslope. When all four cell layers only are included in the section plane, the resultant C/N ratio values produce a straight line; no wave pattern is apparent and the amplitude is zero. This particular gland was sectioned very close to frontal and therefore was close to parallel with the lamellar rows of cells. Since a minimum of two 3 μm thick sections would be required to pass through one 7 μm thick cell layer and the areas analyzed (5000 μm²) each represented a square roughly 10 cells long by 10 cells wide (70 x 70 μm), any slope of the section plane of one in ten or less which would translate as five degrees or less, would keep the area well enough
within the bounds of a single layer of cells to maximize C/N ratios and therefore amplitude. A five degree deviation from squarely frontal is noticeable in histological reconstruction of the RPD of the goldfish pituitary and since the tilt on this gland was probably less than five degrees, the low amplitude of 0.20 probably represents the maximal amplitude for at least this area of prolactin cells. A mechanical explanation, therefore, does not appear to fit this situation.

The functional explanation assumes the laminar architecture of prolactin secreting cells. It was previously shown from examination of the cytoplasmic area (Figure 6) and nuclear area (Figure 5) plots that the nuclear cycle is muted in comparison to the cytoplasmic cycle and that it is basically the area/volume of cytoplasm in cells in the peak regions that determines the height of the peaks. The cytoplasm drives the cycle. The bases of wave patterns are at least similar for virtually all tissue cores examined. In the control gland tissue, vascularization was associated with relatively low amplitude waves compared to those found in avascular regions. It was, therefore, postulated that proximity to vascularization could be equated with stimulation of prolactin secretion, hence, less build-up of storage product in the cytoplasm, subsequently smaller cytoplasmic area and lower peaks than were evident in the avascular
region of the gland. The low waves in the control gland maintained amplitude of about 0.40 as opposed to the 0.20 amplitude in this 24L:0D test animal. It is felt that low level stimulus such as was thought to occur in the control which was supposedly in the steady low-level phase of prolactin secretion, was manifested in those cells near the blood vessels by truncated peaks caused by the absence of prolactin storage in the cytoplasm. High level stimulus could be assumed to result in the secretion of product almost immediately after synthesis. The resultant scarcity of storage product in the cytoplasm would cause a further truncation of amplitude in the wave pattern. This may be what is evident in the core from this test animal. Under constant light, there could be a general stimulatory effect on prolactin secretion, or the animal may have been sacrificed during a diurnal peak of prolactin production. With no dark period and hence no entrainment stimulus, the usually good control over diurnal events may have broken down. With such a small sample, no unequivocal conclusions can be drawn regarding reasons for the apparent high activity. It does appear, though, that these prolactin cells are in a high activity state that is denoted by the low amplitude of those waves. With such a small sample size, however, the possibility that this wave pattern may be a result only of individual variability cannot be excluded.
The trend line of best fit through the 4L:20D ratios (Figure 21) produced a wave pattern that had two well-defined and complete waves with amplitudes of approximately 0.30 and periods of 33 μm and 27 μm. The short periods of the waves can again be accounted for on the basis of the average cell size of that particular population of cells analyzed. Since the first complete wave (33 μm) has a longer period than the second (27 μm) it would appear that the tissue core progressed from an area containing a population of cells in the 8 μm diameter range into a cell population of 5.5 μm to diameter. Actually, the difference in period between the two waves is almost small enough to be able to discount it as being within the limits of experimental error and error in the placement of the trend line-of-best-fit through the data points.

The amplitude of 0.3 which is 25% lower than that of the low amplitude vascular core waves from the control gland, requires further explanation. Of the two most plausible explanations for low amplitudes that were detailed in the immediately preceding analysis of the 24L:0D gland, it is felt that a mechanical explanation is most relevant. Because average cytoplasmic area fluctuations were within the same range (11.57 μm² to 21.34 μm²) as found in the vascular regions of the control gland (7.51 μm² to 21.34 μm²) and this relatively large variation was evident on
histological examination, the functional state of the tissue core was thought to be comparable to that of the vascular core from the control glands and, therefore, a mechanical explanation for the low amplitude was sought. The most immediately apparent cause for the lowered amplitude followed from histological reconstruction of the gland, when it was found to have been sectioned at about a 10 degree lateral tilt from squarely frontal. As explained previously, the combination of such a tilt of the section plane with the length of the field analyzed would result in truncation of both troughs and peaks in an analysis of laminar cell groups. A ten degree tilt of a 70 μm long field of analysis would extend through about two rows of 8 μm diameter cells. Any field measured of the size used in this study would thus be composed of cells in approximately equal proportions from two adjacent regions of the wave form and, since the four phase secretory cycle is thought to produce a sinusoidal wave form it would result in the compression of the wave into a narrower range. For instance, no maximal theoretical peak reading could be obtained because the area measured would always include either ½ peak plus downslope, or ½ peak plus upslope, or ½ peak plus ¼ high upslope plus ¼ high downslope, or some proportion of the preceding.

In wave forms of low amplitude where a similar period is maintained to that of a comparable high amplitude wave,
the slope of the line joining peaks with troughs will be shallower in the low amplitude wave than in the high amplitude wave. As has been shown, or at least theorized, the amplitude of waves is linked either to a functional activity state where higher activity probably means a lower amplitude waveform, or it may be linked to by the angle of section plane through the lamellar cell arrangement. Given the lamellar cellular architecture and sections parallel to the lamellae, it may be possible to determine the "activity state or level" of populations of cells or even large tissue areas from both the amplitude of the waveform and the slope of the lines joining peaks with troughs. One obvious problem with this is that similar effects can be caused by a fairly small tilt in the section plane from parallel to the lamellae. Other than serial reconstruction of the gland to determine degree of tilt, the amount of truncation of, or the rise in value of the C/N ratios of the troughs may provide at least an indication that a low amplitude wave pattern may be due to mechanical rather than functional reasons. The bases of most glands sectioned frontally and therefore parallel to the lamellae show C/N ratios approximating 0.5 whether they are high, medium or low amplitude waves and regardless of period. With the particular type of secretory cycle shown for the prolactin cells, functional activity that results in a truncation of amplitude should not produce
a noticeable effect on the C/N ratio of the troughs. On the other hand and for the reasons already explained, the mechanically caused truncation of wavelengths should result in both lowering of peaks and raising of troughs. Although this effect is not very apparent in the 4L:20D test animal, there is some hint of it in the centre trough and the effect is quite apparent in test animals from the 12L:12D and 0L:24D photoperiod regimes. Again, because of small sample size in the study, these effects cannot be unequivocably substantiated. Since the relationships between all of the variables from secretory cycle, through techniques that combine to produce the wave forms still are not entirely understood, these immediately preceding thoughts are conjectural rather than definitive.

The challenge to the lamellar architecture theory represented by the obvious four-wave pattern of the 16L:8D (Figure 19) pituitary with its regular 15 \( \mu \)m period was initially a complicating factor for the theory, but ultimately added another and interesting dimension to the analysis of the wave patterns. It was immediately obvious from the quite regular 15 \( \mu \)m period and from the number of sections that made up each of the waves, that no four phase and lamellar secretory cycle such as had been proposed, that was composed of cells approximately 7 \( \mu \)m in diameter, would fit into each of the four waves. If two of the waves were
considered together, as parts of a single wave, two of the commonly encountered 30 μm periods could be accounted for from the data generated. Since the second and fourth troughs were the deepest, it was thought that the first two peaks possibly represented one actual complete secretory cycle based wave and the second pair another wave. When this 16L:8D RPD was reconstructed from serial sections, it was found to be the gland that had been sectioned the closest to perfectly frontal of all the glands that were examined. In addition, it was also noted that some serial sections were very "dense" with nuclei and others were not. From the lamellar architecture theory, if the hypothetically perfect case were taken where the cells were all of an ideal, equal size of 9 μm in diameter, nuclei were central and 3 μm in diameter and they were sectioned at 3 μm perfectly parallel to and coincident with the rows of nuclei, a pattern as shown in Figure 45a would be produced where low C/N ratios would coincide with each row of nuclei and infinitely high C/N ratios would coincide with purely cytoplasmic areas. There could be no C/N ratio generated wave pattern. If there were a regular and rational change in the amount of cytoplasm associated with each cell layer where the change goes from a 6 to 3 ratio of cytoplasm to nuclei on the peak, to a 3 to 3 ratio on upslope/downslope to a 1 to 3 ratio on troughs, a pattern such as shown in Figure 45b would be formed. The
measurements used in these examples are not meant to produce an accurate scale model of the study systems, but are meant to illustrate the points being made. If enough scatter of nuclei around the mean, perfectly planar arrangement is introduced to bring C/N ratios from cytoplasmic areas down from infinity, then an approximately bimodal curve with two smaller side peaks, as shown in Figure 45c would be formed. With more randomness of nuclear position within the cells, a smoother curve would be produced similar to that shown in Figure 45d. The wave forms produced by the trend line for this 16L:8D test animal most closely resemble the bimodal example of Figure 45c. They are believed to illustrate the type of wave pattern generated when the area analyzed from a tissue that is arranged in a laminar fashion is very close to parallel with the lamina. From this it would appear that a slight angle to squarely parallel is preferred for the production of smooth wave forms such as have been obtained on most of the analyses. When sections are too close to perfectly parallel with the rows of cells in the various secretory cycle phases, additional extraneous, but real peaks and troughs are generated that mask the overall cycle. It is difficult with the present experience to either predict or to determine the theoretical height of peaks in such waves and therefore possible activity state of the cell population. However, the 16L:8D pattern has illustrated well the sensitivity of the Quantimet analysis technique and has also
Figure 45a-d. Cytoplasmic-nuclear distributions in relation to the production of variability in wavelength appearance.
provided additional support for the postulate concerning
existence of sheets of prolactin cells in lamellar array
associated with secretory cycle.

Taking this analysis of sections which closely parallel
the rows of cells to an extreme, based on limited knowledge
of precisely what may be occurring during the secretory
cycle and of the precision of the present measuring techniques,
some other interesting predictions and models can be proposed.
Since the direction of the cycle can be determined relatively
easily from nuclear and nucleolar morphology, upslope of
these waves can be distinguished from downslope and the waves
can be examined for consistently occurring asymmetries that
might be related to function. For instance if the secretory
cycle is as highly synchronized in a lock-step manner as is
suspected might be the case, the following wave patterns
deduced from what is thought to be known about the cyclical
cellular morphology should be encountered:

(1) In an avascular unstimulated cycle and with all cells
in the middle of the phases of the cycle, troughs
(regeneration phase) should contain almost bare nuclei,
upslopes (synthesis phase) should contain moderate
amounts of cytoplasm, downslopes (release phase) should
also contain moderate amounts of cytoplasm and peaks
(storage phase) should contain large amounts of
cytoplasm. This should produce a symmetrical wave
similar to that shown in Figure 46a.
In an avascular cycle with all cells at the beginning of their phases, troughs should again contain almost bare nuclei, upslopes should contain very small amounts of cytoplasm and peaks should likewise contain moderately large amounts of cytoplasm. This should produce an asymmetrical bimodal waveform in which that half of the wave that contains the upslope will be narrower because there is somewhat less measured cytoplasm in that half of the wave whether the intercellular substances act in a collapsible fashion or as a firm framework while the cells expand and contract. That half of the wave should also have a steeper slope because of the greater nuclear density relative to cytoplasm than will be found in the downslope half of the wave. In somewhat exaggerated form, its pattern should be similar to that illustrated in Figure 46b. Interestingly, the wave forms in the 16L:8D test animal could, with a little imagination, be said to fit the mirror image of the just described form. On the basis of the nucleolar and nuclear cycles, it was determined that the direction of synthesis in the wave was from left to right. In both patterns, the left half of the bimodal wave is wider. One, the first wave appears as if it should fit the slope predictions, but the other is opposite (the usual case with biological systems).
Figure 46a.
Theoretical appearance of a wave pattern generated by cells located in an avascular region and which are in the middle of their respective secretory phases in the cell cycle. Symmetry.

Figure 46b.
Theoretical appearance of a wave pattern generated by cells located in an avascular region and which are at the beginning of their respective secretory phases in the cell cycle. Asymmetry.
So, these 16L:8D waves may fit into the scheme that would propose cells in synchrony at the ends of each of the phases.

(3) It is felt that bimodal waves from areas of high activity would show the same sorts of patterns but would be more compressed.

The above explanations, although theoretical are consistent with previous observations.

The line-of-best-fit through the C/N ratio values of the prolactin cells of a test animal maintained under conditions of total darkness (Figure 22) sloped from 1.0 to 0.70 units in the primary 54 µm of the tissue core. It then ascended from 0.70 to 0.90 units through the remaining 27 µm of tissue. No period could be determined from these results. It was suspected that the slope might represent a very steep tilting of the gland. Due to the "lamellar" arrangement of the prolactin cells within the RPD, the cells in the plane perpendicular to the banding pattern should produce a straight line (C/N ratio) because their phases of the cell cycle would be synchronous. In order to determine the tilt of the gland, a grid of the cellular arrangement of the RPD was constructed. Arbitrary values of 8, 12, 8 and 4 were assigned to the upslope, peak, downslope and trough regions of the model. A second grid represented, to scale, 3 µm serial sections through these cells and could be fitted to
the first grid in a variety of ways in order to reconstruct the angle of sectioning and the number of cells which had to have been sectioned to produce the C/N ratio graph (Figure 22). Seven to ten prolactin cells per width of the Quantimet machine field (63 μm) were usually analyzed in each serial section. By grid, it was determined that a seven cell width had been analyzed by this method through the initial 54 μm of the tissue core. Furthermore, the pituitary gland was found to be tilted horizontally at an angle of 60 degrees off frontal. Microscopic examination confirmed this angle, or degree of tilt. The gradual increase in slope peculiar to the remaining 27 μm of tissue could not be reconstructed from the grid and it is thought to be the result of some kind of distortion caused by the grasping of the tissue by the forceps. This area coincided to the edge of the gland, which is, by virtue of its location, most vulnerable to the many mechanical traumas which can be induced by the dissecting and processing techniques employed. An alternative explanation for this curve involves an arhythmnia of these cells in the absence of light. The first explanation is, however, consistent with the "lamellar model" of the prolactin cell architecture of this region.

The line-of-best-fit through the prolactin cell C/N ratios of the 112 μm core of tissue analyzed from a test animal maintained on a 12L:12D photoperiod possessed
variability in period ranging from 51 \( \mu \text{m} \) to 30 \( \mu \text{m} \) to 21 \( \mu \text{m} \), (Figure 20). Employment of the grid to aid in explanation of this proved difficult. It could be determined that the gland had been sectioned at a 25 degree angle through a width of seven cells. This explained the period and amplitude of the first wave, however, the change in these two parameters in the successive waves could not be clarified by this method. Clues were sought through histological examination. The microscopic appearance of this gland differed greatly from those previously observed. The rostral region of the gland was malshaped. The anterior and lateral regions were elongated and bumpy, rather than contoured and smooth as they had been in other goldfish. This region extended so far in an anterior direction that it gave the impression of being totally "segregated" from the more posterior areas of the pars distalis. It was well vascularized and contained an exceptional number of chromophobes. A section-by-section analysis suggested that the high anterior-posterior tilt of the gland resulted in a very rapid crossing through the bands thereby producing a very complex pattern which could not be analyzed by grid or even theoretically explained.
Corpuscles of Stannius

Corpuscles of Stannius are present in Teleostei and Holostei, but are absent in Dipnoi and Chondrostei. They vary in number among species and frequently, between individuals of the same species. Structurally the arrangement of the tubules and pseudolobules shows interspecific variation and Krishnamurthy and Bern (1969) classified the CS in a number of different fishes into four basic structural patterns.

In C. auratus, corpuscles were of the Type I arrangement which these researchers described as being composed of compact cords lined by a single cell layer along the connective tissue septum. This arrangement resulted in a tubular appearance in cross section and a palisade appearance in the longitudinal axis. The histological observations of the CS in the present study supported this structural description. The electron microscope observations of Oguri (1966) suggested that two basic cell types can be distinguished. The "S1" type contained numerous secretory granules and had a poorly developed RER. The "S2" type contained few secretory granules and a well developed RER. In addition to these he also identified a third cell type ("SM") which he suggested was an intermediate type between "S1" and "S2". He concluded that these stages represented differences in the functional state of the same cell. He further suggested that the presence of an RER as opposed to smooth ER may imply that these cells are involved
in the production of a protein-like hormone, rather than a steroid hormone. Based on early studies, Fontaine (1964), Anguilla anguilla; Fontaine (1967), Anguilla sp., Chan (1968), Anguilla anguilla and A. japonica; and Ogawa (1968), C. auratus; the surgical removal of these glands induced hypercalcemia as well as a marked sodium and chloride loss. These results generally confirmed Oguri's suggestion, based on his own histological observations and the early findings of Fontaine (1964) that these organs might produce a calcium lowering substance which was proteinaceous in nature. The nature of the secretory product and the role of these glands in water and electrolyte balance is described under Review of Literature. More recently, studies concerning the ultrastructure of these corpuscles confirms that they possess the typical features of protein-producing endocrine organs - extensively developed RER, a prominent Golgi complex and the presence of numerous secretory granules (Tomasulo, et al., 1970, Lebistes reticulatus; Cohen, et al., 1975, Fundulus heteroclitus; Fenwick, 1974, Anguilla rostrata; Bhattacharyya & Butler, 1978. Opsanus tau). Generally, a structural diversity of cell types has been described. However, whether this diversity represents functional diversity, or whether it is the result of different phases of a secretory cycle peculiar to one cell type, must be determined with further experimental analysis. Bhattacharyya
and Butler (1978) state that from the structural diversity in cell types observed in the toadfish, it may be suggested that the granule-loaded cells may represent a phase of either secretion or storage. Those cells lacking granules but possessing numerous ribosomes and large amounts of RER, a well-developed Golgi apparatus and with an abundance of coated vesicles in the immediate vicinity, are deemed metabolically active and are suggestive of the synthesis of material. A totally agranular cell type of small size, condensed Golgi apparatus with a few small saccules, scarce amounts of RER, with small-sized cisternae and few ribosomes in the cytoplasm, may represent a cell phase or exhaustion or atrophy. Although this description of cell types or phases is peculiar to the toadfish, the structural diversity of cell types or phases has been generally documented. In addition to the major populations of granulated cells, sparsely granulated cells have been described in C. auratus (Oguri, 1966), the eel (Anguilla japonica), the guppy (Lebistes reticulatus) and the salmon (Salmo salar) and researchers have also observed cell degeneration in the teleost CS (Fujita & Honma, 1967; Tomasulo, et al., 1970; Carpenter & Heyl, 1974; Lopez, 1969; Johnson, 1972; cited in Bhattacharyya & Butler, 1978). Oguri's suggestion of structural diversity of one cell type in C. auratus is therefore credible. Also noteworthy is that the size, shape and granule distribution of most of the
CS cells in the toadfish, guppy, salmon, goldfish, killifish and bowfin show a homogeneity peculiar to each species, but also comparable among these fishes. In other species such as the eel, *Anguilla anguilla*, the stickleback, *Gasterosteus aculeatus*, and the rainbow trout, *Salmo gairdneri*, two cell types have been described and show differences in granular size and shape and other cytological features (Krishnamurthy & Bern, 1969; Wendelaar Bonga & Greven, 1975; Wendelaar Bonga, et al., 1977; cited in Bhattacharyya & Butler, 1978). Their functional roles, however, must be defined experimentally.

In the present study, one centrally-located tissue column comprizing one whole corpuscle was examined. Changes in the morphology of the cells along this core were evident from the regular changes in cell area (Figure 30) which resulted in area-based wave patterns of relatively consistent amplitude and period. There is a fairly even distribution of data points along the curved trend line. Approximately 25% of all points within this data set can be assigned to either peak, trough, upslope or downslope regions of the wave. It is therefore suggested that the four distinguishable phases comprizing the wave are the result of morphological changes indicative of a four phase secretory cycle. Although these data were obtained from a static specimen it is felt that the continuity of the wave patterns generated from section by
section analysis of the gland, coupled with documentation from literature of the possibility of cell secretory phases in C. auratus, permits a dynamic interpretation of a secretory cycle although the dangers inherent in this process are fully acknowledged. Histological support for a cyclical secretory pattern was acquired through examination of the cells comprizing each of the four phases of the area-produced waves. The majority of cells that correspond to peak regions (Figure 31) possess an abundant amount of cytoplasm believed to contain secretory product. The nuclei were plump, round to oval or indented and were basally to centrally located. No prominent nucleoli were observed. Chromatin was diffuse and/or clumped. It is thought that these cells are in a storage state. Alternatively, they may also be slowly releasing their product or may be engaged in concurrent synthesis and release of the hormone hypocalcin. The majority of cells that correspond to trough regions (Figure 32) appear to have scant amounts of cytoplasm. Nuclei were small and round through oval or of an irregular shape. Nucleoli were frequently encountered and multi-nucleolar conditions were observed in some nuclei. Infrequently, cells possessing two small nuclei were noted. Chromatin was diffuse. This variability of morphology is suggestive of cells in either rejuvenation or else in preparation for active synthesis of a proteinaceous product, primarily through the production of ribosomes. The
μm² in trough regions, while intermediate values of 17.91 ± 3 μm² and 17.14 ± 3 μm² occurred in upslope and downslope regions respectively (Table 9). These changes are suggestive of a cyclical manufacture and release of product. When nuclear areas and changes in nuclear appearances are considered in relation to the changes in cytoplasmic area, strong indications of cyclic direction result.

It is suggested that the direction of this cycle is in an antero-posterior direction along the corpuscle, which depicted in Figure 30 in the cell area graph, runs in a left to right direction. As in the case of the pituitary, the recognition of peak and trough regions is more easily achieved than the discernment of upslope and downslope regions.

The Wave Pattern

The cell area-produced wave patterns in Figure 30 resulted from a sample population of approximately 5,000 cells. In addition to this data, exhaustive histological examination of the morphological changes occurring within the sample population suggests that individual hypocalcin secreting cells undergo a cyclical activity which can tentatively be divided into four phases i) synthesis, ii) storage iii) secretion iv) reorganization and preparation for synthesis.
Nuclear Area

The distribution of values for the nuclear area data set (Figure 25) suggests an irregular four-wave pattern which approximates the cell area-generated four-wave pattern (Figure 30). Although the data points fluctuate, their general distribution does allow some prediction of peaks and troughs, upslopes and downslopes in the cell area graph. Therefore, changes in nuclear area seem to be fairly good predictors of the various phases of the cell cycle. It also seems that nuclear area contributes largely to the phasing of the cell cycle. In other words, the nucleus appears to be going through a cycle which precedes the corresponding cytoplasmic cycle. The approximate correspondence of nuclear area with total cell area, in the case of the C S, is thought to be at least partially due to what was previously referred to as "vagaries in technique". The use of area in this case gives rise to some inter-section variability probably due to differential flattening of tissue. This may account for the area fluctuations between individual data points. However, it is not enough to disrupt the overall cyclical pattern observed for this parameter.
Cytoplasmic Area

No readily discernible pattern resulted from the distribution of these data (Figure 26) and they could not be related to histological changes in any consistent manner. When this plot is compared to the cell area-produced wave pattern (Figure 30), there appears to be very little difference in cytoplasmic areas between peak and trough regions, although in some instances, the largest cytoplasmic areas do approximate the position of the peak regions of the cell area graph. These data were, therefore, not useful for indicating changes taking place in the cytoplasm of the cell. This was not expected since histologically, it appeared that nuclear and cytoplasmic area changes were somewhat directly related - i.e. nuclear area enlargement seemed to be closely followed by an increase in cytoplasmic area. However, if the slight fluctuations observed between each data point in the graph of nuclear area were due, in part, to inter-section area change as the result of differential flattening, similarly it is possible that the same fluctuations might mask or disrupt the pattern of cytoplasmic area changes that may be present. Evidence for cytoplasmic area changes was obtained by histological examination of the cells in peaks, midregions and troughs of the cell area graph.
The distribution of data points (Figure 27) did not show any discernible pattern in fluctuation which could be correlated to histological changes indicative of a cell cycle. Interestingly, however, when the cytoplasmic area graph, (Figure 26) is compared to the C/N ratio graph (Figure 27), the area data points fluctuate in the same directional point-for-point pattern with the ratio data points. In contrast, the nuclear area data set (Figure 25) moves generally in a point-for-point direction opposite to the ratio data points. The changes in area of nuclei and cytoplasm, although reflected in ratio, are masked or muted by this means of expression. This results from the morphological changes which occur in these cells during the manufacture of secretory product. In this instance, the nuclear changes precede those of the cytoplasm. The nucleus enlarges in area, or is "active" prior to the change in cytoplasmic area. It seems that these changes are not entirely direct in relation to each other, but that a slight staggering in change occurs between the nucleus and cytoplasm. The ratio data fluctuations indicate to some extent how these two cellular components are changing in relation to each
other, but the overall effect is one of masking and/or muting and ratio is, therefore, not a good predictor of the various phases of the cell cycle which are more clearly demonstrated by area measurement.

Cell Area

The plot for cell area (Figure 30) shows a distribution of data points in the form of four wavelengths. This graph was used to interpret cyclical changes associated with the cell secretory cycle. Histological examination of the peaks (Figure 31), troughs (Figure 32), upslope (Figure 33) and downslopes (Figure 34) confirmed the presence of morphological changes which could well be correlated to a cyclic pattern suggestive of a secretory cycle. It is the fluctuations in nuclear area that is largely responsible for the area-produced wave pattern. When nuclear and cytoplasmic areas are combined in cell area, the lag between the two components, as well as the slight staggering as the area of each increases, is minimized and the resultant areas are representative of the nuclear-cytoplasmic morphological changes which occur during the secretory cycle. However, if the lag between the two cellular components is readjusted (i.e. the nuclear area data set [Figure 25], is shifted back three data point positions) in relation to the data for cytoplasmic area (Figure 26), a comparison between the two areas illustrates
that the maximum area values for each generally approximate the peak regions of the cell area graph. Similarly, the same is true for trough values and the overall correspondence between the cytoplasmic, nuclear and total cell area graphs (Figures 25, 26 and 30 respectively) is maximized. This information is important as it clarifies, to some extent, the inter-fluctuations or inter-phase changes which were previously termed "staggering". Subsequently, when lag is considered and corrected for, the morphological changes between cytoplasmic and nuclear area become more regularized. Conversely, when lag is not considered and the real activity of these cells is dealt with, it is possible, with the aid of the preceding explanation, to better understand the nuclear-cytoplasmic interactions which occur and why, when each parameter is considered separately, it is the nuclear area graph which approximates the cell area plot. This is considered to be the best predictor of the various phases of a cell secretory cycle. Area proved to be the analytical parameter most suited to express the relationship between changes in nucleus and cytoplasm inherent in a cell cycle wherein the nuclear changes precede those of the cytoplasm - i.e. wherein the nuclear changes seem to drive the cycle - as is the case with the C.S. This area-produced wave pattern (Figure 30) served as a guide in the location of cells in the various secretory phases through histological
methods. On the other hand, ratio (Figure 12) best expressed these relationships in the instance of the prolactin cell of the pituitary gland. Herein, a very different nuclear-cytoplasmic interaction took place and the changes in cytoplasmic area were largely responsible for the production of a ratio-produced wave pattern which, likewise, permitted the accurate location of cells in any one of the four phases of the secretory cycle. In this latter case, the cytoplasmic area plot (Figure 6) approximated the ratio-produced wave patterns (Figure 12a, b), but the interaction between the cytoplasm and nucleus of cells during the different phases of the cycle was such that these phases were best defined by ratio which had a regularizing effect on these nuclear-cytoplasmic fluctuations.

The second important difference between these two glands and, therefore, in the type of parameter which best expresses their cell cycle, is that in the case of the pituitary, comparable cell populations are comprised of cells of different sizes and the use of area in this case would be misleading because, for example, a 40 \( \mu m^2 \) cell may be in the same secretory phase of a secretory cycle as one which is 24 \( \mu m^2 \). However, the phases become comparable when expressed as C/N ratio. This does not seem to be the case in the C.S. All area values for cells lie within reasonable boundaries and there is nothing to suggest that comparable (i.e. peak-
peak) cell populations are composed of cells of different sizes. In fact, so little fluctuation in area is seen between comparable regions of the wavelengths that when confidence limits are placed on the line of best fit, only ten of the 61 data points lie outside the boundaries.

An interesting problem arose in the analysis of the initial C.S. analyzed. As stated previously, with the use of area as an analytical tool, caution must be practiced. When the cell area graph (Figure 30) was first plotted, a difference of 8 \( \mu \text{m}^2 \) was noted between sections 1-28 and sections 29-61. These serial sections occurred on two different slides. If an adjustment of 30\% was made, i.e. the cell areas on the 2nd slide were raised the corresponding difference, the curves generated for the gland fit together. Since there appeared to be no good reason to expect that a 30\% difference between the average areas of two adjacent sections which contained portions of the same cells was valid, the adjustment was deemed justifiable. The 30\% difference is probably not an optical one because the difference in thickness between the two slides was 5/1000 inches, (this should produce no effect on magnification) the difference in thickness of balsam and coverslip was 2/1000 inches, (this could account for some small effect) and the difference in total thickness of both slides was 1/1000 inches. The error could not be attributed to the machine nor its operator since
the sequence in which the sections were done was: sections 1 to 19, 36 to 61 and 20 to 35 with one week intervals between measurements and with no analysis of results until the complete data set had been gathered. Since the sections were cut in one continuous microtome run and were passed through the staining series in the same boat, it is thought that the likeliest possibility for error production lay with the flattening and affixation procedure. Mayer's albumen (dilute) was spread on the slide, the serial sections arranged on the fluid and the slide was then placed on the warming tray (37°C). The sections were then allowed to flatten, were positioned, the excess fluid drained off and then dried. Since the slides were treated separately in this process, it is possible that differential flattening and spreading of the sections probably occurred during this process just by leaving one set of flattening sections on the warming tray longer than the other, so it spread further during the flattening process. The resultant difference of 30% certainly makes the use of direct area and/or volume measurements on cells suspect, especially when taken from different slides and probably even from serial sections from the same slide, because there is no real guarantee that adjacent sections have not flattened differentially especially if they had originally suffered differential compression during cutting. That such a uniform wave pattern emerged from the comparison of average cell areas
in spite of the distortion that can so easily be produced by the manipulations of technique attests to the overriding presence and strength of the pattern imposed on the tissue by the synchronized secretory cycles of the cells.

In sections 1-12, the distribution of data points shows greater fluctuations in cytoplasmic and nuclear area and therefore in C/N ratio (Figures 25, 26 & 28) than is seen throughout the data points representative of the rest of the gland. Sections 1-12 corresponded to the edge of the gland. Here, the pseudotubules exhibit a randomness of orientation. Many small nuclei were observed within this region of the gland. This accounted for a decrease in average nuclear area. This same randomness of tubular orientation also resulted in serial sectioning of cells in such a way that nuclei were not present in all cells. This naturally caused a large increase in average cytoplasmic area. However, as the pseudotubules became aligned in parallel array (sections 13-61) this problem was solved and more consistent values for each data set were obtained.

The wave pattern indicated by the trend line through the points generated from the average cell areas (Figure 30) of approximately 150 cells per area analyzed shows a remarkably uniform period of approximately 42 \( \mu \text{m} \) and an amplitude of about 8 \( \mu \text{m}^2 \). The average cell diameter is approximately 8 ± 2 \( \mu \text{m} \). Most of the larger cells are found on the peaks with
the smaller ones in trough areas. The gland was sectioned at 3 μm and since the cells are relatively tightly packed and are fairly uniformly polygonal in shape, it would require about three sections to encompass one cell. For instance, points at sections 44, 45 and 46 produce the peak of the wave between sections 40 and 50; sections 41, 42 and 43 produce the upslope; 47, 48 and 49 comprise the downslope and 50, 51 and 52 and 38, 39 and 40 produce two troughs. This would indicate that the banding pattern is probably produced by a one-cell-thick layer of cells that are in different phases of a secretory cycle. It is postulated from the preceding information that the pattern must be the result of a secretory cycle in which the cells are filling up with secretory product and then secreting it, but are doing so in synchrony with a band of other cells that extends much of the way across the gland. How this synchronization is maintained is at present unknown, but it can be speculated that mechanisms such as tight junctions and/or concentration gradients might produce such a result. If the interpretation of the wave pattern is correct, to reiterate, it means that the secretory cycle is composed of four phases - storage (peak), release or secretion of product (downslope), repair and regeneration (trough) and synthesis (upslope). Since there are roughly equal numbers of points on each phase of the cycle, it may be that each phase is of an equal, but as yet, undetermined
duration. If that were so, it would provide a mechanism that could lock in the synchrony of the system. Such a lock mechanism would also ensure a supply of the hormone at all times.

Analysis of Tissue from Test Animals from Other Photoperiods.

A sample size of n = 10 serial sections located in a central position of each corpuscle from the remaining test animals was taken. Cell area was plotted against serial section number. In each instance, regression analyses of area with serial section number was characterized by lines of variable slope (Table 12). Correlation coefficients were not significant (p = 0.05) in eleven of the thirteen attempts. Examination of the data from each test animal suggested, however, that in numerous cases, data points occurred in "clusters" which were somewhat suggestive of a cyclical distribution pattern, above and below the least squares line of best fit. However, it was also obvious that the sample size was too small to produce one wavelength and therefore, comparisons between the area changes evidenced by the test animals and the "control" were not possible. At least one full wavelength is necessary for meaningful comparisons of changes in cell activity reflected in the changes of amplitude and/or period which could be correlated to some change in the cell secretory cycle. Furthermore, as previously stated, in the control animal, a 30% difference
between the average cell areas of tissue on two adjacent
sites resulted from differential flattening of this tissue.
Therefore, the differences in areas observed for cells from
the test animals may again be the result of differential
flattening. To further complicate any interpretation of the
area data, all tissue from each test animal was mounted on a
separate slide. Variable amounts of balsalm were used to
affix coverslips to these slides and therefore, it is feasible
that a magnification difference could also have been induced.
For instance, when all these factors are considered, a cell
area of 32 \( \mu m^2 \) may be representative of a trough area cell in
preparation for synthesis. At the same time, from a different
slide, a cell area of 26 \( \mu m^2 \) may be representative of a peak
cell in a storage phase of the cycle. These data then, if
taken at the face value of their area measurement, can lead
to a very erroneous conclusion in relation to cell phase of
activity during the cycle. Due to the preceding, area
measurements were an unsuitable method of comparing any
cellular changes which took place within the corpuscles of
the test animals maintained under the various photoperiodic
regimes.

As evident in the "control" animal (12L:12D) cell area
(Figure 30) best expressed the cyclical morphological
changes inherent in the secretory cycle. However, rather than
lose the remaining experimental data, it was decided that
these data, when expressed in ratio, should be indicative of any changes occurring between cells of various test animals. Expression of the data in ratio form would also virtually eliminate the errors induced by differential flattening of tissue and/or magnification errors and the tissue on the slides could be compared. Mean ratio values were plotted for males only, females only and for males plus females versus their respective photoperiod regime. This graph is depicted in Figure 35. Mean C/N ratio values for males only showed an upward trend in relation to the increasing number of light hours to 12L:12D whereupon, these values decreased. However, the 16L:8D value (0.99) was not too different from that at 8L:16D (0.90). At 24L:0D, the ratio value (0.84) was similar to those at 2L:22D (0.86) and 4L:20D (0.85). These data suggest that there is an increase in cellular activity that seems to be related to an increase in the number of light hours. This increased activity is at its maximum in males at 12L:12D photoperiod. At either extreme of photoperiod and at photoperiods with a small number of hours of light (i.e. 2L, 4L) the C/N ratio values are very similar. If this maximum of cellular activity at the 12L:12D regime is not peculiar to the test animal itself, it may be that the C.S. cells are responding to an increased demand for a calcium lowering substance at this photoperiod. Alternatively, photoperiod may be directly influencing these cells and in males the
maximum stimulation-response occurs at the 12L:12D photoperiod.

The C/N ratio values for females increased to 4L:20D (0.98) and then remained fairly stable through to 12L:12D (0.93). At 16L:8D a sharp increase (1.18) occurred followed by a drop at 24L:0D (0.90). The latter value is similar to that at 2L:22D (0.85). Unlike the case of the male, the maximum C/N ratio value for the female occurred at 16L:8D. Similarly, the female may be responding to an increased demand for a calcium lowering substance at this photoperiod, which is not as critical to the male and may be related to the influence of photoperiod (lengthening light hours) on the female's reproductive cycle. Alternatively photoperiod may have its greatest effect on the cells of the C.S. in females at the 16L:8D photoperiod. From the plasma calcium graph (Figure 3), it can be seen that generally both males and females can closely regulate their plasma calcium levels over a wide range of photoperiods, namely those most often encountered in temperate zones. No correlation between glandular activity and plasma calcium levels can be made. However, the activity of the C.S. increases with photoperiod. It may be possible that there is a direct photoperiodic effect on the cells of the C.S. Alternatively, this component of the calcium-regulating system may be responding to some variable whose axis can interact with this system at specific photoperiods, which places a greater demand on these cells for a calcium-lowering hormone (i.e. estrogen).
Ultimobranchial Gland

Oguri (1973) examined the ultimobranchial glands of goldfish collected at different months of the year. He described these glands in immature goldfish, collected during August, October and December as being small and composed of cell cords. Those fish collected during the spawning season showed hyperplasia of ultimobranchial tissue, formation of tubules and a hypertrophy of the cells lining these tubules. The goldfish in the present study were winter fish and the appearance of their ultimobranchial glands were similar to the fall and winter fish in Oguri's investigation. However, there was less hypertrophication of the entire gland.

The histological appearance of these glands in the goldfish approximates more closely the description by Yamane and Yamada (1977) for the ultimobranchial glands of the Masu salmon, Oncorhynchus masou. Likewise, the description by McMillan, et al., (1976) for the ultimobranchial of the rainbow trout, Salmo gairdneri, is similar to that of the goldfish in the present study. Investigators such as Yamane and Yamada, (1977), McMillan, et al., (1976) and Deville and Lopez, (1970) describe the gland as having a pseudostratified epithelium which is arranged in follicles or cords surrounding a lumen, cavity or cyst. Although these papers show photomicrographs in which red blood cells are located...
in the lumens of these cysts, follicles, cords and lobules, the lumens are not described as either sinuses, venules or capillaries. In the present study, no lumen of any ultimo-branchial "cyst", follicle, cord or lobule was noted without a discernible endothelial cell lining.

In all the glands examined, cells of a variety of morphological types were evident. The previously described cell categories probably represent cells in various stages or phases of a secretory cycle. To reiterate, what will be referred to as Phase 1 would be represented by the cuboidal-eosinophils and the columnar-eosinophils with slight basophilic cast. The cuboidal eosinophilic cell would correspond to a stage preceding the initiation of secretory product production. The columnar eosonophil with the basophilic cast would correspond to a stage in which RNA synthesis begins and ribosomes accumulate in the cytoplasm. This would be expected to precede protein synthesis. Phase 2 would be represented by columnar basophilic cells and the columnar basophils with clear apices. The basophilic cell would represent the state of a maximum accumulation of ribosomes in the cytoplasm followed by the beginning of the accumulation of the secretory product in the clear apex of the cell. Phase 3, represented by the larger flask to round-shaped cells, would correspond to a state of continued accumulation of secretory product in the cytoplasm and the
possible storage of that product. Phase 4, the "deflated" cell of slightly basophilic cast, would correspond to cells that have released or are in the process of releasing their secretory product. The type of product release is thought to vary from merocrine through apocrine to holocrine. Some of the very large cells in the acini appear to break down completely in a holocrine form of release. Yamane and Yamada (1977) noted this form of release in addition to what they referred to as "exfoliation" of the epithelial cells in the Masu salmon.

There is considerable support for such a secretory cycle in the literature. Some authors have assigned a storage function to similar large clear cells (Deville & Lopez, 1970; McMillan, et al., 1976). Robertson (1967) proposed a secretory cycle for the frog, Rana pipiens, ultimobranchial which was very similar to that noted in the goldfish. Deville and Lopez (1970) show a somewhat similar series of changes that occur in the salmon, Salmo salar L. ultimobranchial gland during the course of migration.

McMillan, et al., (1976) in their immunohistological studies on the ultimobranchial gland of the trout, Salmo gairdneri, showed that a very large proportion, but not all epithelial cells contain calcitonin. They concluded that the high concentration of calcitonin in the trout ultimobranchial gland is attributable to large amounts of hormone
stored in these endocrine cells and to the high concentration of endocrine cells in the ultimobranchial gland epithelium. Assuming the categorization of the cell morphologies into four phases or stages that can plausibly be related to function, detailed examination of cell profiles from individual photoperiods should permit some tentative correlations of overall glandular appearance with functional state. At 0L:24D and 2L:22D photoperiod regimes, the preponderance of epithelial cells in the eosinophilic-cuboidal and columnar-eosinophilic stage with the remaining 30% clear cells which appear to be in a storage phase and are located in round acini below the epithelium, indicate an inactive gland. The storage phase clear cells were considered to be in a true storage phase since all were turgidly plump, and none extended up into the epithelial layer through the necks of the acini. Although the gland appears to be completely inactive (relative to release of secretory product) its appearance alone is not enough to rule out the possibility of a low level of secretory activity. At 4L:20D, the gland shows a low level of activity as is evident from the presence in the epithelial layer of roughly a 50-50 proportion of cuboidal and columnar-eosinophils (Phase 1) and basophilic (Phase 2) cells. Approximately 30% of the total number of cells observed were clear cells in flask-shaped acini with prominent neck region into which these clear cells extended.
At 8L:16D the gland shows an increased level of activity as is evident from the number and appearance of basophilic Phase 2 cells. Similarly, approximately 30% of the total number of cells observed were clear cells in flask-shaped acini with neck regions into which these clear cells extended. Half of the clear cells were plump while the other half were fusiform in appearance. At 12L:12D, the gland has a more active appearance than that of 8L:16D as is evident from the appearance of the basophilic Phase 2 cells. These cells were very high columnar and many had clear apices. The fusiform-shaped clear cells, which comprised 25% of the total cell population, extended up into the necks of the many flask-shaped acini. At 16L:8D, the gland is again considered to be highly active, but generally gives the overall impression of being spent. The Phase 2 basophils appear elongated and "greyish". Acini generally contain a few plump clear cells, but most are deflated. The clear cells (Phase 3) represent 26% of the cell population. Occasionally a Phase 4 cell is seen. At 24L:0D, the gland is similar in appearance to those at 0L:24D and 2L:22D. However, the epithelial cells are more generally of columnar-eosinophilic (Phase 1) appearance and the possibility that they are involved in a slow rate of protein synthesis cannot be eliminated. Clear cells in acini (Phase 3) are again located deep to the eosinophilic epithelium and, similarly to the case in all other photo-
periods, represent 28% of the cell population.

In light of the four-phase secretory cycle theorized for both prolactin cells of the rostral pars distalis and the epithelial cells lining the tubules of the Corpuscles of Stannius, it is interesting to note that in the ultimo-branchial gland, clear cells maintain a relatively constant proportion (25 - 30%, Table 15) of the cell population in spite of the apparently different activity levels of this gland at the various photoperiods. This crude measure lends indirect evidence and further credence for the existence of some synchronizing mechanism which maintains a roughly constant proportion of the cells in each phase of the secretory cycle. This perhaps implies that somehow, under normal conditions, the capacity to be able to react to the demand for secretory product is maintained while enough reserve product enables any further demands on this system to be immediately met.

If the extremes of photoperiod are excluded from analysis, there seems to be a correlation between photoperiod and the histological appearance of the gland, with low, apparent activity corresponding to short photoperiod, high activity with a "normal" photoperiod and overactivity with slightly longer than "normal" photoperiods. These animals were sampled ostensibly near a uniform time after the onset of light and thus, if a diurnal cycle of activity in these glands exists, the histological results should be
TABLE 15.

Proportions of Clear Cells versus Other and the Apparent Activity of the Gland at the Various Photoperiods.

<table>
<thead>
<tr>
<th>PHOTOPERIOD</th>
<th>CELL COUNTS (Clear vs Other)</th>
<th>APPARENT ACTIVITY of the Gland</th>
</tr>
</thead>
<tbody>
<tr>
<td>0L:24D</td>
<td>30% vs 70%</td>
<td>Inactive</td>
</tr>
<tr>
<td>2L:22D</td>
<td>30% vs 70%</td>
<td>Inactive</td>
</tr>
<tr>
<td>4L:20D</td>
<td>31% vs 69%</td>
<td>Low activity</td>
</tr>
<tr>
<td>8L:16D</td>
<td>30% vs 70%</td>
<td>High activity</td>
</tr>
<tr>
<td>12L:12D</td>
<td>25% vs 75%</td>
<td>High activity</td>
</tr>
<tr>
<td>16L:8D</td>
<td>26% vs 74%</td>
<td>High activity (exhausted)</td>
</tr>
<tr>
<td>24L:0D</td>
<td>28% vs 72%</td>
<td>Inactive</td>
</tr>
</tbody>
</table>

comparable. No-one has investigated diurnal histological activity of the ultimobranchial gland. If there is no diurnal cycle of activity, then the results of the present study indicate the possibility of a histological response related to photoperiod. A correlation between increased activity of this gland and the reproductive cycle in fishes is documented (Oguri, 1973; Deville and Lopez, 1970; Yamane and Yamada, 1977). In C. auratus, gonad maturation usually takes place in those months characterized by lengthening photoperiod (Oguri, 1973). Therefore, interactions between increased
glandular activity, photoperiodic effect and the onset of reproductive activity in these fish cannot be ruled out. However, in the present study, none of the fish which had been acclimated to the longer number of light hours exhibited visual signs of gonadal maturation, although histological work was not done to confirm this observation. Nevertheless, a histological response related to photoperiod was evident. At the extremes of photoperiod, these glands are of similar morphology. It may be that the effect of both total dark and total light is equally disruptive on "normal" function of the ultimobranchial gland. If there is a correlation between photoperiod and glandular activity, and light is considered to be a stimulus, it follows that none, or too few light hours may render the gland insensitive. At the opposite extreme, total light, the gland may exhibit the same appearance due to an over-stimulation which, likewise results in an insensitivity to the stimulus.
Correlation Between U.B. Gland Morphology and Plasma Calcium Levels

There appears to be a correlation of the visual appearance of the ultimobranchial glands with photoperiod. The apparent increase in activity of this gland cannot however be correlated with the plasma ionic calcium level profiles. At photoperiod 0L:24D the appearance of the gland is one of relative inactivity; plasma calcium levels are low (♀, 1.9 mM⁻¹). The full-looking, clear cells (stored product) in the gland are all concentrated in the acini below the cuboidal and columnar epithelium. A few neck regions of the acini are apparent penetrating the epithelium. The acinar basal cells are also cuboidal and uniform. The glands from test animals maintained at 2L:22D are almost identical in appearance to those of the 0L:24D specimens - the glands appear inactive. Plasma calcium levels are low (♀, 2.2 mM⁻¹; ♂, 2.0 mM⁻¹). Glands from fish at photoperiod 4L:20D show some activity. There is a general increase in height of the epithelium with many columnar and a few flask-shaped with prominent neck regions and basal cells that are cuboidal to high cuboidal. This heightened activity corresponds with blood calcium levels of ♀, 2.3 mM⁻¹; ♂, 2.5 mM⁻¹, which are both higher than in the two previous photoperiods. 8L:16D glands show a high activity profile in which the epithelium contains cells of all stages and so many of the long flask-shaped, clear cells
that protrude down into the body of the gland that the epithelium does not appear continuous. Acini are quite variable in shape with clear cells in all stages of inflation and deflation. This active appearance of the gland corresponds with plasma calcium levels which are virtually identical to those of the previous photoperiod (4L:20D). 12L:12D glands are indistinguishable from 8L:16D glands, except that there appear to be fewer fully distended clear cells in the acini and more of fusiform shape. This active gland again corresponds with virtually unchanged plasma calcium levels (2.5 mM\textsuperscript{-1} in both the females and the male specimens). The 16L:8D glands also appear active but show quite a different profile in that very few of the large clear cells that probably represent a storage phase occur and most acini contain fusiform-shaped clear cells that are not vacuous. The surface epithelium is also more definite with few clear cells but large numbers of columnar cells. Plasma calcium levels again remain stable ($\varpi$, 2.6 mM\textsuperscript{-1}; $\varnothing$, 2.6 mM\textsuperscript{-1}) at values very close to those from the three previous photoperiods. The 24L:0D glands appear relatively inactive and are quite similar to those from the 0L:24D and 2L:22D photoperiods. Plasma calcium values are lower ($\varpi$, 2.4 mM\textsuperscript{-1}; $\varnothing$, 1.9 mM\textsuperscript{-1}). Plasma calcium levels, in the case of the females, do not drop significantly (although this is difficult to state categorically in the face of such a small sample size - n = 4).
However, in the case of the males, the drop in plasma calcium levels is significant and is within the range of the plasma calcium levels exhibited by those test males at 0L:24D and 2L:22D photoperiods (the same problem occurs at this photoperiod extreme as only three and four individuals respectively are represented). The stable plasma ionic calcium levels found over what can be considered the "normal" number of light hours that would realistically be encountered by a fish in a temperate region (4L:20D - 16L:8D) are within the normal range for the closely related carp species held on a 12L:12D photoperiod (Houston, et al., 1970), but are considerably lower (2.5 vs 3.5 mM⁻¹) than those found by Fenwick (1975) in 35 g goldfish.

It is apparent from these observations that: (a) although there seems to be a relationship between photoperiod and the various histological profiles of the goldfish ultimobranchial gland, there is no correlation between these two parameters and plasma calcium levels: (b) in this population of C. auratus no sexual dimorphism was obvious in either plasma calcium levels or in the histological appearance of these glands at the various photoperiods.
CONCLUSIONS

(a) **Plasma Ionic Calcium**

This study provided no evidence of photoperiodic influence upon plasma ionic calcium levels in presumably sexually immature goldfish maintained under ecologically-realistic photoperiod conditions. Evidence of regulatory imbalance under abnormal photoperiods (i.e., total or near-total darkness, continuous light) was obtained.

(b) **Pituitary**

The observations of the present study can be summarized as follows: (1) individual prolactin cells appear to undergo a form of cyclical activity broadly divisible into four phases: synthesis, storage, secretion, reorganization and preparation for synthesis; (2) from dorsal to ventral regions of the main mass of the rostral pars distalis there exists a well-defined periodicity of phases of apparent activity; (3) phasic periodicity is apparently unaffected by degree of vascularity. Phasic amplitude, on the other hand, may be reduced in relatively well vascularized areas. The latter may be the result of an "overlapping" of the various secretory phases wherein, for example, the synthesis phase could be extended through the normal storage phase and product release or secretion may also be extended back through the storage phase and possibly into the synthesis phase. This would account for such amplitude differences as those
which appear in Figure 12a (avascular) and 12b. In this system, the output of product over a base level could be variably controlled by the strength and/or length of a "prolactin releasing factor" stimulation without changing the period of the system. The amplitude can probably only be lowered to a limited extent by the same method. A "prolactin inhibiting factor" (Schreibman & Holtzman, 1975), may affect the synthesis of product in a negative manner, but again not interfere with the basal cycle of the cells. This could produce a similar attenuation of the amplitude of the wave, yet not interfere with the synchrony of the entire tissue.

The prolactin cells which are locked into secretory step with neighbouring cells, produce an architecture which has been described as a "lamellar model" in the present study. This lock-mechanism may be achieved through gap junctions or diffusion gradients.

Examination of the wave patterns from the various photoperiods suggests that approximately 25% of the cells are maintained in each phase of the secretory cycle at any one time. This would ensure a supply of hormone at all times.

Attempts to determine the "activity state or level" of these cells from the various photoperiods given the lamellar cellular architecture, should theoretically be possible by
comparison of the amplitude of the waves. However, as shown, the degree of tilt at which these glands were sectioned was capable of causing truncation of peaks and troughs and altering the slope of the line joining the two. Therefore, the effect of photoperiod in these cells cannot be stated with any great certainty. However, the cells from the 24L:0D test animal do show a high activity state when compared to the control animal held at an 8L:16D photoperiod. McKeown and Peter (1976) demonstrated that longer photoperiods caused greater release of prolactin from the pituitary of goldfish. The results of the present study basically point to this conclusion as well, however, due to the small sample size, the lack of knowledge of all the variables from the secretory cycle and the problems which can be caused by a small tilt of the gland, the conclusions must be tentative and await further clarification through experimentation.

(c) **Corpuscles of Stannius**

For the initial corpuscle analyzed, the wave pattern generated from the average areas of groups of synchronous cells has been interpreted to mean that the cells possibly exhibit a secretory cycle that can be represented by the four phases of storage (peak), release or secretion of product (downslope), repair and regeneration (trough) and production or synthesis (upslope) of what is assumed to be
secretory product. Since there are approximately equal numbers of points on each phase of the cycle, it may be that each phase is of equal duration. If that proved so, it would provide a mechanism that could lock in the synchrony of the system and ensure a supply of hormone at all times.

The mean C/N ratio values for cells from the test animals at the various photoperiods suggest that photoperiod does produce an effect on this gland, as what is assumed to be "increased cellular activity" is demonstrated over what is considered to be a "normal" range of light hours. However, there is little evidence to suggest that the sexual dimorphism which is known to occur in calcium metabolism in fishes, is reflected in the dimorphism of cellular activity of the two sexes apparent with lengthening of photoperiod. Although this gland is hypocalcemic in nature, no decrease in plasma ionic calcium levels was observed. Clarification of these observations requires further experimentation.

(d) **Ultimobranchial Glands**

The histological appearance of these glands suggested that cells of a variety of morphological types were evident. These morphologies were thought to be representative of the various phases of a secretory cycle. The maintenance of a relatively constant proportion of clear cells in spite of apparently different activity levels of this gland at the
various photoperiods is suggestive of a synchronizing mechanism which maintains a roughly constant proportion of the cells in each phase of the cycle. There seems to be a direct correlation between photoperiod and the histological appearance of the gland, with low apparent activity corresponding to short photoperiod, high activity with a normal photoperiod and overactivity with extended but "normal" photoperiod. Although this gland is hypocalcemic in nature, no decrease in plasma ionic calcium was observed. Clarification of these observations requires further experimentation.

(e) Evidence pointing to the lamellar organization of prolactin- and hypocalcin-secreting cells in the rostral pars distalis and Corpuscles of Stannius respectively - heretofore not described - raises several questions regarding the mechanisms which initiate and maintain secretory functions which warrant further investigation.


Levenstein, I. 1939. The cytology of the pituitary gland of two varieties of goldfish (*Carassius auratus* L.) with some reference to variable factors in the gland which may possibly be related to the different morphological types. Zoologica, 24 : 47-60.


APPENDIX

1. Bouin's Fixative

Saturated aqueous picric acid 75 parts
Formalin (10%) 25 parts
Glacial acetic acid 5 parts

2. Ehrlick's Alum Haematoxylin

Haematoxylin 6.0 gm
Absolute ethanol 600.0 ml
Glacial acetic acid 60.0 ml
Glycerine 600.0 ml
Distilled water 600.0 ml
Alum ammonium sulphate 200.0 gm

3. Dr. Bowie's Eosin

One gm alcoholic eosin in 100 ml, 80% ethanol
One gm yellowish eosin (Y) in 100 ml distilled water
One gm bluish eosin (B) in 100 ml 30% ethanol

4. Cleveland-Wolfe Stain (Modified)

<table>
<thead>
<tr>
<th>Staining Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ehrlick's Alum Haematoxylin (1:1 dilution with distilled water) 10 minutes</td>
</tr>
<tr>
<td>5% aqueous Erythrosin 45 minutes</td>
</tr>
<tr>
<td>1% alcoholic (70% ETOH) Orange G 3 minutes</td>
</tr>
</tbody>
</table>

5. Mayer's Albumen (Stock)

| Fresh egg white 1 part |
| Glycerol 1 part |
| Thymol 1 crystal |
6. **The Quantimet 720 Computer Image Analyzer**

Image analysis is a means of extracting quantitative geometric, densitometric information from images (Swenson & Attle, 1979). In the last fifteen years, the development of the contemporary image analyzer has proceeded at a rapid pace. Advances in electronic and computer technologies have facilitated this growth. Further development has been stimulated by the increasing number of applications of the Quantimet 720 Computer Image Analyzer to problems which demand greater speed in their analysis, higher resolution and/or increased measurement capabilities while maintaining an ease of operation.

Industrial research and development, quality control, biological, clinical and biomedical research are but a few of the disciplines which have benefited from the employment of this instrument.

**Mode of Operation**

Figure 47 depicts a block diagram of the Quantimet 720 Image Analyzing Computer (Imanco).

Briefly, the mode of operation is as follows:

1. a microscope presents an image of a specimen (input) to the image analyzer
2. the scanner, or video camera, converts the optical image into an electronic image
(3) processing instructions are executed within the central processing module; this results in the extraction and treatment of the desired data

(4) output is calculated and may be recorded manually or by means of a teletype

Accuracy of measurement is determined by the accuracy of the imaging, scanning and detection processes. The accuracy of the latter depends upon contrast between the cell particle, etc., and the background, the size of the cell/particle compared with the size of the field of view and the resolution, uniformity and geometrical accuracy of the imaging device and scanner.

Imanco has developed a special flat field microscope.
which is capable of providing accurate geometric and densitometric data. To be useful in image analysis, this equipment must possess a superior optical system consistent with objectives in order to image the microscope fields onto the face of the scanner tube. Apochromatic objectives are employed. Of particular importance are optical shading and light source stability. Uniform and constant illumination is critical. The Quantimet 720 imaging system possesses a matrix shade corrector as well as a highly stabilized 100 watt illumination power supply which ensures through these combined features a superior optical output.

The Quantimet scanner, or video camera, provides the highest practical resolution for quantitative image analysis. This is achieved by combining a high vertical resolution of 720 effective scan lines with high horizontal resolution of 896 resolvable picture points per line. The scan is digitally controlled, which ensures that each scan line and every picture point is not only identical in dimension, but also precisely positioned. 720 lines are scanned sequentially 10.5 times per second. This results in equal resolution of square picture points in both directions. Resolution of the scanner is defined as the number of picture points it can resolve in the field of view. This, coupled with the
modulation transfer function enables accurate definition of the boundary of that which is being analyzed. Subsequently, scanner resolution is the critical limit to overall system resolution.

As previously stated, the information from the vidicon is digitized. One picture point is equal to one digital unit electronically. A scan line consists of a number of these placed end to end. The entire area being scanned is equal to 500,000 picture points. Useful contrast is obtained at as little as one picture point.

Sizes, size distribution, areas, perimeters, numbers, diameters and intercepts in values which can be calibrated can be determined rapidly - one tenth of a second for a complete field. The high resolution of the scanning system allows objects to be accurately distinguished by their light transmission or reflectance properties. Approximately 30 shades, ranging from white through grey to black are available for assignment to the pre-selected features which are to be measured. Image data is analyzed by the integral computer.

**Image Analysis Accuracy**

Quantitative image analysis of the preceding parameters and the production of accurate data is, of course, highly dependent upon the video scanning process. Electrical noise in the form of small random variations in the output signal
affects count, measurement and grey level separation. This limits the capabilities of an image analysis system and can cause a wide random variation in results. However, this can be minimized by the use of the automatic integration of the results from ten successive fields.

Counting and measurement errors which result from noise are incurred by alterations in the grey level threshold. There is no ideal, sharp cutoff line, rather, there occurs a band in which detection threshold cutoff is gradual. Figure 48a (Imanco) depicts an ideal noiseless scanner output signal as a scan line crosses a light feature in a darker grey background. In the absence of noise, the signal from the feature allows accurate counting and measurement at the correct threshold setting. Figure 48b depicts a situation wherein noise causes the electrical output to vary in such a way that at any given instant a signal may be found above or below the ideal signal line. However, the feature in this case is still fairly well defined. The probability of error in counting and measuring is directly related to the magnitude of the noise present; as noise increases (Figure 48c) accurate detection becomes difficult. Due to the multiple grey phases available for assignment to an image, the detection and separation of these phases becomes problematic since each grey level signal possesses its own noise. If the noise pulses overlap, inclusion of the noise
Figure 48a-c. (a) Ideal Noise, (b) Low and, (c) High Noise Scanner Output Signals illustrating the effect of noise on measurement accuracy.
pulses from one grey level may be included in the measurement in another grey level. Noise not only causes undesired features to be detected, but also causes features to vary in size around their correct value. The Quantimet 720 minimizes noise by reducing the scan rate from the commercial television 60 frames to 10.5 frames per second. This permits the distinction of image features which are separated by only 10% of the black-to-white range, thereby ensuring accuracy of analysis.

Scanner sensitivity is important in three respects. First, high sensitivity to light allows distinction of features at low light levels such as those which might be encountered in high magnification electron microscopy. Second, good differential sensitivity enables the scanner to distinguish between features of similar light intensity. Finally, uniform sensitivity of all points of the scan is critical in the production and subsequent analysis of an evenly illuminated image. Quantimet scanners exhibit as little as 6% sensitivity variation over an image containing 500,000 picture points and, consequently, can guarantee this feature (Imanco).

The correction of shading is critical as non-uniformity can result in features of the same grey level being detected or measured in one part of the field, but not in another part. A small amount of shading can result in inaccurate
measurement even when detection of the required feature occurs all over the field of view. Optics, electronics and illumination systems contribute to this error. Figure 49 (Imanco) depicts the effect of shading on feature sizing.

![Diagram showing effect of shading on feature sizing](image)

Figure 49. Effect of Shading on Feature Sizing.

It is obvious that large errors in analysis can be induced through variation in grey levels due to shading. The Quantimet 720 possesses an automatic matrix correction system which makes possible the detection of grey level variations
of as little as 3% across an entire field (Imanco).

Geometric distortion of images results from lenses and scanners producing directional magnification differences for an object as well as variability in magnification of the same object in different positions in the field of view. This results in random errors in measurements related to feature location in the field. However, if a sufficient number of fields is measured, an error in area may average out to a negligible size. Other measurable parameters - i.e. size distribution - cannot be corrected for this error. To avoid distortions of this type, the Quantimet 720 has a ± 0.3 percent scan linearity (Imanco). The outstanding analytical capacities of the Quantimet are the result of a system designed for superior resolution, low noise and outstanding grey level discrimination. Moreover, these attributes enable accurate repeatable results.

Application of the Quantimet 720 to the Present Study

In the present study, the Quantimet 720's capabilities were applied to a histological problem. Total area of cytoplasm and nuclei, total area of nuclei and the number of nuclei, presumably cells were measured. Prior to analysis of the study material, machine threshold values were set for both white and black. The Quantimet was then programmed to maintain an even illumination of the field of view. The
cells to be analyzed were viewed by light microscope under oil immersion. The optical image was then converted into an electronic image visible on the scanner or video camera. As the full field of the scanner was employed, the biological material being observed was comprised of a 500,000 square picture point image. As stated previously, approximately 30 shades of grey (or light transmission) can be recognized by the Quantimet. Thus, in light microscopy tissue studies, it is possible to distinguish the individual cellular components, e.g. nucleus and cytoplasm, as well as the different cell types and the cellular matrix solely on the basis of differential staining. Therefore, the Quantimet 720 is capable of analyses of various histological features through densitometric methods. This instrument has five possible channels which can be preset, therefore, it was possible simultaneously (1) to see and reject all "extraneous" material other than prolactin-secreting cells, or those cells lining the tubules of the Corpuscles of Stannius. This was accomplished by assigning a shade-black to these structures. This designation effectively eliminated unwanted features from even being considered by the Quantimet for analysis; (2) to see and measure the projected area of the desired cells by designation of a shade of grey to them; (3) to see and measure the projected area of nuclei through another shade of grey; (4) to see and "tag" all nuclei present in a field of view and to subsequently compute cell
number through the electronic detection of these "tags".

Data was generated in picture points for the desired parameters analyzed. For example, the area of cytoplasm plus nuclei might equal 404559 picture points. The area of nuclei might be comprised of 230747 picture points. And, the cell numbers for that particular field of view might equal 148. Calibration of the picture points in $\mu m^2$ suggests that 10 pp $= 1 \mu m$. It is then possible to convert the data obtained into $\mu m^2$ and establish average cytoplasmic and average nuclear areas, as well as their ratio. The following is an example of this procedure:

**Average Cytoplasmic Area :**

$$\frac{173812}{100} \text{ pp} = 1738.12 \mu m^2$$

$$\frac{1738.12 \mu m^2}{148} = 11.74 \mu m^2$$

**Average Nuclear Area :**

$$\frac{230747}{100} \text{ pp} = 2307.47 \mu m^2$$

$$\frac{2307.47 \mu m^2}{148} = 15.59 \mu m^2$$

**C/N Ratio :**

$$\frac{11.74 \mu m^2}{15.59 \mu m^2} = 0.75$$

The same procedure was repeated for each of the sections
analyzed. The accuracy of the Quantimet succeeds the accuracy of making successive sections. In order to compensate for slight variations in section thickness or staining intensity, the threshold values were adjusted for each section in a manner normal to the operation of the equipment. This, of course, is a source of operator bias inherent in the method of measurement. This bias is, however, much less than that inherent in alternative computation techniques.

To acquire optimal results from the application of this methodology in an investigation of this scope, required that great care be taken to ensure that serial sections were of a uniform and appropriate thickness. Flattening, staining and coverslipping of the material was standardized in an attempt to eliminate variables which could interfere with the comparative analyses of the test tissues.

The Quantimet 720 is a powerful analytical tool and its application to histological problems has introduced quantification into a discipline which heretofore has been restricted to observation and tedious, incomplete attempts at quantification.

In much of the literature pertaining to this study, evaluations of endocrine function (activity) was based on either a descriptive approach, which included subjective assessments of cell to cell differences, or else, such studies assumed that individual secretory cells were randomly
distributed in relation to activity and therefore, measurements made at random were used as indicators of glandular activity. Quantitative analysis of these glands by the Quantimet 720, in this thesis, suggests that the latter is not necessarily the case.