THE FUNCTIONS OF THE FISH PINEAL ORGAN

by

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ABSTRACT

The role of the fish pineal organ has been studied using the goldfish *Carassius auratus* and the Pacific salmon *Oncorhynchus tshawytscha*. To this end, the effects of pinealectomy in goldfish on various behavioural responses, endocrine systems, and the reproductive system were studied. The pineal organs and the retinal tissue from mature and immature salmon were examined by thin-layer chromatography and fluorometry to determine if melatonin, a mammalian hormone, is present in the fishes. Goldfish were injected with melatonin to see if the effect of exogenous melatonin was opposite to that of pinealectomy.

Pinealectomized goldfish lost the photo-negative response seen in normal goldfish. Blinding had the same effect on phototaxis as pinealectomy and a combination of the two had the same effect as blinding or pinealectomy alone. It was concluded that the normal phototactic response depended upon both the pineal organ and the eyes. Pinealectomy, blinding, or both was followed by a marked increase in swimming activity. Although this increase was correlated with a decrease in the whole brain serotonin level, a causal relationship was not established between the two. Further, pinealectomy alone produced no significant changes in whole brain serotonin level.

Melatonin was localized within the pineal organ of salmon and its concentration in this tissue was analyzed. The pineal melatonin store varied during the reproductive cycle and was
found in lower concentrations in the pineal organs of mature salmon. Stored melatonin could not be found in the retinal tissue despite evidence for an active tryptophane metabolism in this tissue.

Injection of melatonin into goldfish inhibited the increase in gonad size under long photoperiod; this was accompanied by larger gonadotrophs in the melatonin injected fish. Removal of the pineal organ from goldfish held under short photoperiod caused an increase in gonad size similar to that seen in untreated goldfish exposed to long photoperiod. The effect of pinealectomy on the gonads was limited to that season during which the gonads could be stimulated by increasing day length. At other times of the year, neither photoperiod nor pinealectomy produced any significant effect on the gonad size. From this it was concluded that the pineal gland of the goldfish is related to the reproductive cycle and that its function depends upon photoperiod and the production of melatonin.

Pinealectomy had no effect on the interrenal tissue, thyroid tissue, plasma osmotic concentration, or plasma levels of Na⁺, Cl⁻, or Ca+++ indicating that the effects of this operation are specific for the reproductive system.

The data obtained from these studies support the hypothesis that the pineal organ of fishes serves a secretory as well as a sensory function. Further, the functional aspects of the mammalian and fish pineal organs are discussed and it is concluded that the role of the pineal organ is similar in the two groups; that is, the pineal organ of mammals and fish is involved in the
timing of reproductive events.
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Extensive and illuminating bodies of literature concerned with the ontogeny, morphology, histology and cytology of the fish pineal organ are readily available. Nonetheless, no basic or primary function has been unequivocally demonstrated for the pineal organ of fishes. This failure to reveal a basic function of the pineal in fishes probably stems directly from the paucity of physiological experiments concerning its role.

Despite the numerous reports of physiological effects of pinealectomy in other animal groups, relatively few workers have examined the effect of pineal ablation on fishes. Further, although the hormone melatonin has been localized in mammals, birds, and amphibians, and although the enzyme necessary for its synthesis has been found in the pineal organs of reptiles and fishes, nobody has demonstrated the presence of melatonin within the fish pineal. Moreover, the effects of exogenous melatonin have not been described in fishes despite the marked physiological effects of this substance on other vertebrates.

The aim of this investigation, then has been to examine these problems and to extend our knowledge of the function(s) of the fish pineal organ.
Although the pineal organ of mammals has assumed a position of respectability among the endocrine organs (Thiéblot, 1965), this same organ of fishes is viewed by many as a functionless, evolutionary vestige of a primitive third eye. This schism probably arises from the obvious morphological differences between the photosensory like structure of the fishes and the glandular appearance of the mammalian pineal organ. The pineal of fishes strongly resembles a sensory structure with recognizable photosensory cells (Studnička, 1905; Holmgren, 1959a; and Rüdeberg, 1966) and afferent nerve fibers running from the pineal organ to other parts of the brain (Hill, 1894; Kappers, 1965; Rüdeberg, 1968a, b; a.o.). Conversely, this same structure in mammals has a glandular appearance (Kitay and Altschule, 1954; Relkin, 1966) with no primary sensory cells and efferent innervation only (Kappers, 1965). The efferent fibers described rarely in the fish pineal (van de Kamer, 1955; Hafeez and Ford, 1967), though possibly functional, are probably of the same type of aberrant commissural fibers described in some mammals (Kappers, 1965). Despite the differences in the way that the information reaches the organs, the pineals of fish (Grunewald-Lowenstein, 1956) and mammals (Quay, 1956; Zweig et al., 1966) show responses to external illumination.

Friedrick-Freksa (1932), Rasquin (1958), Holmgren (1959b) and Hafeez and Ford (1967) have now presented evidence that the secretory activity of the fish pineal organ occurs within the
sensory elements of the pineal epithelium. Rüdeberg (1968a), though unable to educe any definitive evidence for secretion within the sensory cells of the pineal organ of _Sardina pilchardus sardina_ (Risso), reported some evidence for an autocoidal role of these cells. This possibility of a sensory and a secretory function of the same cells was supported by Vivien and Roels (1967) who observed certain cells within the chelonian epiphysis which possessed the ability to change to and from a receptor and secretory effector. Thus, as pointed out by Roth (1964), it is possible that the phylogenetic trend toward a more glandular structure in the mammals, as opposed to the sensory pineal organ of fishes, reflects a change in the way information reaches the pineal rather than a complete and unrelated change in function. It was therefore speculated that the pineal organ of fish is both sensory and secretory and is functionally similar to the pineal organ of mammals.

In pursuance of these suppositions, the working hypotheses listed below, and discussed in their appropriate section, were tested.

(i) The pineal organ of fishes, by virtue of its photosensitivity, mediates behavioural responses to environmental illumination.

(ii) The pineal organ of fishes synthesizes melatonin and this substance has a hormonal function.

(iii) The pineal organ of fishes is associated with the endocrine system, the reproductive system, or both, and this association(s) is influenced by environmental
illumination.
SECTION I

THE SENSORY ROLE OF THE PINEAL ORGAN IN GOLDFISH

CARASSIUS AURATUS L.

INTRODUCTION

The pineal organ of the cyclostomes has been recognized as a photoceptor since Young (1935) demonstrated that the marked diurnal rhythm of color change in lampreys was abolished in ammocoetes and disturbed in adult Lampetra following pineal extirpation. Breder and Rasquin (1950), Hoar (1955) and Schonherr (1955) report pigmentary dispersion following pineal occlusion or destruction and attribute a photoreceptor function to the teleost pineal. Dodt (1963) and Morita (1966) substantiated this conclusion by electrophysiologically detecting alterations in nervous activity in the pineal of Salmo irideus during and after illumination.

Studies on the effects of pineal occlusion and ablation, blinding, or combinations thereof have provided diversified and contradictory results when behavioural parameters were used. Breder and Rasquin (1947) reported that in a series of blind Mexican cave characins Anoptichthyes the sign of phototaxis was reversed from positive to negative when an otherwise exposed pineal organ was covered, and that exposing an otherwise covered pineal reversed the sign of phototaxis from negative to positive. These same authors reported that the phototaxis demonstrated, whether positive or negative, depended upon the presence of
intact optic cysts with nervous connection. These findings were not corroborated by studies on other fish (Hoar, 1955; Pang, 1965).

Hoar (1955) found that the normal photo-negative response of sockeye salmon smolts *Oncorhynchus nerka* was not obliterated by blinding alone but that the fish did become indifferent to light when the blinded animals had their pineal areas damaged by probing. The data from animals with intact vision but with the pineal area probed were equivocal but did suggest an indifference to light on the part of the animals. Hoar (1955) was hesitant however and did not draw this conclusion. Pang (1965) has more recently reported that blind *Fundulus heteroclitus* show a significantly greater preference for light than the light indifferent blind plus pinealectomized *Fundulus*. Unfortunately, he provides no information about the normal phototactic response of wholly intact animals under similar experimental conditions so that it is impossible to draw conclusions about the effect of blinding alone, pinealectomy alone, or a combination of the two on the phototactic response of this species. Although these studies have implicated the pineal organ in responses to illumination, they have not examined the possibility that the differences resulted from changes in sensitivity to factors other than light or to changes in the general activity of the animals. Further, these workers have not recognized that phototaxis depends upon two things; that is, a phototactic response depends on a preference for a particular degree of illumination and the "readiness" of the animal to move from one condition of
brightness to another. Indeed, Janzen (1933) found that when goldfish were given a choice between light and dark, some preferred the light, some preferred the dark, while others were intermediate in their preference. On the basis of these results he suggested that two features were concerned in the phototactic response; one, a preference to remain under the same degree of brightness which he termed a "persistence tendency" and two, the seeking out of a preferred level of brightness. He also showed that these two features were anatomically separated within the brain (Janzen, 1933).

Recently, Wurtman (1967) has suggested that the effects of photic information in higher vertebrates may be divided into three categories: (1) the psychological effects of a visual field which may cause active orientation or behaviour to a particular visual image; (2) short term neurological reflexes over which the animal has little or no control and which cause passive responses such as regulation of pupillary size and kinésis; (3) and certain long term neuroendocrine responses such as the maturation of gonads. The pineal organ of fishes may be involved in any or all of these effects. Therefore, the present study was designed to test the effect of pinealectomy on both features of the phototactic response, and on the ability of the pineal to operate as a photoreceptor governing active or passive responses to environmental illumination. In addition, the effect of pinealectomy on the response of goldfish to other environmental stimuli and swimming activity was tested. Further, as the pineal organ of fishes (unpublished data) was
found to have the highest serotonin concentration of any region of the brain, and as this substance has been related to the degree of behavioural sedation (Brodie and Bogdanski, 1964; Quay, 1965a; Weisman, 1967), the relationship between pinealectomy, blinding, total brain serotonin and swimming activity was studied.

This section reports on the effect of pinealectomy, blinding and pinealectomy plus blinding on the distribution of goldfish in a light gradient, ability to use light and sound as conditioned stimuli, amount of swimming activity, and total brain serotonin levels. Also investigated were the effect of pinealectomy on the minimum electrical voltage required to just produce a detectable response and the effect of pinealectomy on the distance that goldfish move following stimulation with a constant voltage. The data are then discussed in relation to the possible sensory role of the goldfish pineal organ. The possible role of the pineal organ in long term neuroendocrine responses to photoperiod will be considered in Section III.
MATERIALS AND METHODS

Goldfish (Carassius auratus, common variety; 7.5-10 cm size class) were obtained from the Goldfish Supply Company, Stouffville, Ontario. Excluding the actual time of observation, the fish were maintained in nylon-screen baskets approximately 20 cm square which were suspended in a large holding tank (approximately 400 liter capacity) containing dechlorinated water. The pH of the water in the holding tank varied from 6.4 to 6.9 and the holding temperature ranged from 11 C to 12 C. An artificial photoperiod of 8 hours of light alternating with 16 hours of darkness was maintained at all times; the lights came on at 8:00 AM and went off at 4:00 PM. The goldfish were fed Clark's New Age dry fish food at 9:00 AM daily, with the exception that fish were not fed on the day of testing.

Unless specifically mentioned, six groups of animals were compared in each experiment: pinealectomized (P), sham-pinealectomized (S), and unoperated controls (C), blind (B), blind plus pinealectomized (BP), and blind plus sham-pinealectomized (BS). The first three classifications are occasionally referred to collectively as the eyed groups and the remainder may be considered as the eyeless groups.

Operative Procedures
A. General

Fish were anaesthetized in tricane methanesulphonate (MS 222, Sandoz; 1:1,000) until incapable of righting reactions.
The area of operation was illuminated with a bright microscope lamp sufficiently removed to prevent undue heating of the fish. Following the operation, the gills were irrigated with oxygenated water until spontaneous gill movements reappeared.

B. Pinealectomy and Sham-pinealectomy

Following narcotization, the fish was wrapped in a damp paper towel. The area of the pineal was exposed by making a "U" shaped cut in the parietal bone with the center of the U bisecting an imaginary line connecting the posterior margins of the orbits. The resulting bone flap was lifted towards the top of the U; this was done carefully to avoid breaking the skin on the intact side of the U. If the flap of bone broke free the animal was destroyed. Under a dissection microscope (16X), the pineal gland was detached with a gentle suction applied through a glass pipette. Only those animals seen to lose their pineal organs were considered pinealectomized. Animals in which the brain was damaged during the aspiration of the pineal organ were discarded. After pineal ablation the flap of bone was returned to its original position and the wound was covered with a water impermeable paste (Orabase, Squibb Home Drugs Division, Montreal, Quebec). This paste adhered to the surface of the head for about 24 hours and was thought to reduce leakage of water into the skull cavity. No evidence for residual or regenerated pineal tissue was found in any of the pinealectomized goldfish when examined histologically or during dissections up to four months post-operatively. Sham operated controls were treated in
a similar manner except that the pineal organ was not removed.

C. **Blinding**

While narcotized, the fish was wrapped in a moist paper towel. Curved forceps were used to pull the eye partially free from its socket and the ocular muscles, nerves and connective tissue were severed with a scalpel. Following the operation, blind fish resumed feeding within one half of an hour and appeared to remain healthy.

D. **Post-operative Treatment**

Following the various operations, the animals were held in a 0.2% solution consisting of equal parts of CaCl₂ and NaCl to which Terramycin (Animal Formula, Pfizer Co., Montreal, Quebec) had been added to the extent of 250 mg to each 100 liters of solution. All experimental animals and controls were held under this condition for a period of one week before they were transferred to the original tanks.

Under the preceding regimen, animals remained healthy and mortality was less than 2% in any of the experimental groups.

**Apparatus and Experimental Procedures**

A. **Phototaxis**

The distribution of goldfish in a light gradient was observed in three tanks. Each tank was 224 cm long, 25 cm wide, and 25 cm deep and was filled to a depth of 10 cm with dechlorinated water which contained 1 gm of NaCl/liter. The tanks
were constructed of galvanized iron and were painted grey. Experiments were conducted in a darkened room; one end of each tank was illuminated with a 60 watt incandescent light set 30 cm above the surface of the water. The water temperature within the tanks varied between 14 °C and 16 °C but no temperature gradient was found along the length of any one tank, nor between any two of the tanks.

For each test group, 10 goldfish were used. Observations consisted of counting the number of goldfish in that half of the tank nearest to the light. Ten counts were made during one day, then the light was moved to the opposite end of the tank and ten counts were taken the following day. The light was left on continuously. In this way, 20 observations were obtained for each experimental treatment. Fish were placed in the trough during the evening of the day previous to observation.

B. Conditioning to Light and Sound

Goldfish were subjected to a conditioning situation similar to that described by Agranoff (1967). The aquarium used was constructed of clear plexiglas with the sides covered by black plastic to prevent the fish from observing external movements; the fish were observed in an overhanging mirror. The aquarium was 44 cm long, 11 cm wide and 14 cm deep and the inside was partially divided by a central barrier 10 cm high fixed to the floor. Water was added to the depth of 12 cm and provided 2 cm of water over the central barrier. One gram of NaCl was added to each liter of water to facilitate the conduction of electrical
currents. Each half of the tank was provided with a pair of stainless steel electrodes (12 cm x 10 cm) and an unshaded 40 watt bulb was placed at either end of the tank. The bottom of the tank was covered with 2 cm of sand but in the middle of the tank the sand on both sides of the barrier was banked up to the top of the barrier. This arrangement of the sand provided a clue for the blind animals which otherwise had difficulty in avoiding the central barrier during the conditioning experiments. A suitable series of switches permitted the lights to be turned on or off at either end of the tank and provided a means by which a series of electrical impulses could be discharged between either pair of electrodes. The series of electrical shocks was provided by a Grass S6C Stimulator (Grass Instruments, Quincey, Mass.) and consisted of seven pulses of 80 volts per second for 20 seconds; each pulse had a duration of 1 msec. A learning trial began with the illumination of the light located on the same side of the central barrier as the fish. After 20 sec, a series of electrical shocks was initiated in the same end, and were allowed to continue for a further 20 sec at which time both the light and the shocks were terminated and a 20 sec rest period ensued before a similar sequence was initiated at the opposite end. Fish which did not swim over the barrier in response to the light alone or to the light plus the shocks were given a one minute rest period before the sequence was repeated.

Each fish was given 50 trials, and five fish from each group were tested. The total number of times that each fish escaped the shock by swimming over the barrier in response to
the light was recorded.

Conditioning to sound was carried out in a manner similar to that described above. Animals naive to the experimental situation were used but in place of a light, a doorbell was provided as the conditioned stimulus. The bell was turned off as soon as the fish crossed the barrier.

Fish did not cross the central barrier without experiencing the unconditioned or conditioned stimulus as this required the fish to bring their dorsal surface out of the water. Therefore, the results obtained were not due to random movements over the barrier.

Both experiments were run in a controlled environment room at a temperature of 12 C and in subdued light.

C. Response to Electrical Stimuli

Responses to electrical stimulation were measured in a trough similar to that used by Elson (1942). The trough was 130 cm long, 12 cm wide, and 15 cm deep and was constructed of plexiglas. Sufficient water containing 1 gm of NaCl per liter was added to the tank to bring the depth of water to 8 cm. The long sides of the trough were covered with black plastic and the trough was arranged so that an overhanging mirror could be used to observe the fish. Experiments were carried out in a room uniformly illuminated with a dim light. The bottom of the tank was marked with transverse black lines into equal segments 2.5 cm wide. The lines were readily seen in the mirror, and each line was assigned a number from 1 to 52. Stainless steel
electrodes measuring 8 cm by 10 cm were placed in the water at opposite ends of the trough. A switch through which a capacitor could be discharged was wired to a voltmeter so that the voltage used could be recorded (Hoar et al., 1955). Single fish were placed in the trough around 4:00 PM of one day and were first tested at 8:30 AM of the following day. In this way the fish were given a short period to become accustomed to the experimental condition. The problem of diurnal variation in swimming activity was averaged out by subsequent testing at 12:00 noon and 3:30 PM.

Commencing with a subthreshold voltage, stimulation was carried out at 30 sec intervals while increasing the voltage 1 volt at a time until the fish showed its first perceptible response; this voltage was recorded as the minimum response voltage. The procedure was repeated five times with a two minute rest between each trial. The stimulus strength was then increased to the voltage used as the unconditioned stimulus in the light and sound conditioning experiments (80 volts). The fish, provided that it was still, was then stimulated at one minute intervals and the distance that the fish moved during the initial dart was recorded. Ten such recordings were obtained during each of the three daily test periods. At the end of the day, the 15 recorded minimum response voltages, and the 30 recorded distances were averaged separately to give the average minimum response voltage and the average distance moved for that fish.

Extensive variability was expected from the experimental
procedure employed (Elson, 1942; Hoar et al., 1955). For this reason, temperature and photoperiod were controlled. All fish were tested in the same apparatus, one fish being tested each day. Values from five pinealectomized, five sham-pinealectomized, and five intact controls were obtained in this manner.

D. Amount of Swimming

The amount of swimming by goldfish was observed in a circular channel with an outside diameter of 116 cm and with the channel 10 cm wide and 20 cm deep. The circumference at the middle of the channel was 307 cm. Water was 10 cm deep and for the purpose of experimental uniformity 1 gm of NaCl was added to each liter of water. The channel was constructed of galvanized iron and was painted grey. The bottom of the channel was marked with eight equally spaced transverse black lines. Swimming activity was measured in the same manner as used by Hoar et al. (1955) by recording the total number of lines crossed by three fish during three 10 minute intervals. To adjust for diurnal variation in activity, recordings were taken in the morning about 8:00 AM, at noon, and in the afternoon about 4:00 PM. Fish were placed in the channel at 4:30 PM the day preceding the day of observation. Observations were made on five groups of three fish from each of the six experimental classifications. One group of fish was tested each day. The lights in the room were turned on at 8:00 AM and turned off at 4:00 PM.
Serotonin Levels in Whole Brains

A. Preparation of Tissue

Fish were killed by transection of the spinal cord just above the posterior border of the opercula. Whole brains, minus the pineal organ and optic nerves, were removed and weighed to the nearest 0.5 mg. The brains were then placed in a 10 ml Thomas Tissue Hand Homogenizer (Arthur H. Thomas C., Philadelphia, Pa.) containing 0.1 ml of 3% ascorbic acid in 1% dipotassium ethylenediamine-tetraacetate (EDTA-K₂, Cambridge Chemical Products, Detroit, Mich.). Sufficient 0.02 N HCl saturated with KCl was added to bring the final volume of fluid, including the tissue fluid, up to 3 ml. For this purpose, the tissue was judged to be 75% water and the serotonin assessed to be in the water phase (Anden and Magnusson, 1967). The brains were homogenized with a teflon pestle driven by a power stirring apparatus and the homogenized samples were stored separately in polyethylene bottles over solid CO₂ for no longer than three days.

B. Fluorometry

Prior to fluorometry, the brains were thawed individually and washed twice with diethyl ether. The serotonin levels were then determined by measuring the native fluorescence of 5-HT in 3N HCl (Udenfriend et al., 1955; Bogdanski et al., 1956) following the differential extraction of the amine (Quay, 1963; Wise, 1967a, b) in n-butanol. Fluorometry was performed with a Turner Model 111 Fluorometer (G.K. Turner Associates, Palo Alto, Calif.) fitted with a #110-855 far ultra violet lamp and with the
appropriate primary and secondary filters to permit activation with 295 mu and measurement of the fluorescence at 550 mu. To reduce the variability, the fluorescence of each sample was measured in the same round, non-fluorescent, quartz cuvette which had been so marked that it was possible to place the cuvette in the fluorometer in the same position each time. With this arrangement, the polarizing filters suggested by Wise (1967 a) were not required. The total brain serotonin level was determined from each of five fish from each of the six experimental conditions tested in the swimming activity experiment. All serotonin levels were calculated from a standard curve obtained by extracting various concentrations of serotonin standard.

C. Reproducibility, Extraction Efficiency and Specificity of Serotonin Determinations

The brain homogenates of 11 separate goldfish were pooled, mixed, and then divided into 11 equal volumes. A known amount of serotonin (0.20 ug) was added to four of the samples. Identical amounts of DL-5-hydroxytryptophane, 5-hydroxyindole-3-acetic acid and N-acetylserotonin were added to three other samples; one indole in each sample. The remaining four samples were left in their native state. All indoles were purchased from the Mann Research Laboratories, N.Y., and were thin layer chromatographically pure.

On each of four successive days, one native sample, and one to which serotonin had been added were analyzed. The
serotonin content of identically treated samples tested on different days were compared to establish reproducibility of the technique. The percent recovery of serotonin was calculated by subtracting the amount of serotonin determined in the untreated sample from the value obtained after addition of serotonin, dividing the sum by the amount of serotonin added and multiplying the quotient by 100. For this purpose, values obtained on the same day were used in the calculations. Related compounds were all tested on the first day and percent recoveries were calculated. The values so obtained were used to indicate the specificity of the technique.

The results from this check are summarized in Table I and indicates the excellent reproducibility obtained. There was slight variability in the extraction efficiency (85%-95%) and the mean percent recovery of 91% was slightly lower than reported by Quay (1963) or Wise (1967a). The activation/fluorescence maxima of 295/545 mu is characteristic of many 5-hydroxy- and 5-methoxy-indole compounds (Udenfriend _et al._, 1955; Quay, 1963), and it is probable that some of the fluorescence obtained from the native samples resulted from compounds other than serotonin. The 10% recovery of DL-5-hydroxytryptophane, although lower than reported by Quay (1963), is still sufficient to introduce some error into the accuracy of the method. Nevertheless, serotonin comprises the largest fraction of brain 5-hydroxy- and 5-methoxy indoles when the above procedure is used (Wilhoft and Quay, 1965). Further, the values reported herein for the goldfish approximate those reported earlier.
<table>
<thead>
<tr>
<th>Day</th>
<th>Sample (ug added)</th>
<th>5-HT Determined (ug)</th>
<th>Recovery* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>brain</td>
<td>0.42</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>brain+5-HT</td>
<td>0.61</td>
<td>95</td>
</tr>
<tr>
<td>1</td>
<td>brain+DL-hydroxy-tryptophane (0.20)</td>
<td>0.45</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>brain+5-hydroxy-indole-3-acetic acid (0.20)</td>
<td>0.41</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>brain+N-acetylserotonin (0.20)</td>
<td>0.42</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>brain</td>
<td>0.39</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>brain+5-HT (0.20)</td>
<td>0.56</td>
<td>85</td>
</tr>
<tr>
<td>3</td>
<td>brain</td>
<td>0.41</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>brain+5-HT (0.20)</td>
<td>0.60</td>
<td>95</td>
</tr>
<tr>
<td>4</td>
<td>brain</td>
<td>0.41</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>brain+5-HT (0.20)</td>
<td>0.59</td>
<td>90</td>
</tr>
</tbody>
</table>

*%·recovery equals b-a/c x 100.
(Bogdanski et al., 1963). Thus, the error introduced by interfering compounds was considered small enough to render the results valid. To check the extraction procedure, known concentrations of DL-5-hydroxytryptophane and blanks were concurrently and similarly treated with tissue samples within each series. The effective sensitivity of this technique was 10 ng per sample of standard solution though the accuracy and efficiency of the technique was greater at higher concentrations.
RESULTS

Pinealectomy, Blinding, and Phototaxis

Figure 1 summarizes the effect of pinealectomy, blinding, or both on the phototactic response of goldfish. Unoperated control fish and sham-pinealectomized fish were negatively phototactic. Removal of either the pineal organ or the eyes resulted in a uniform distribution in the light gradient. If it is considered that an overlap between the 95% confidence intervals of two groups indicates nonsignificance of the difference between means, and that no overlap indicates a significant difference at the 0.05 level, the differences mentioned above are significant. The apparent positive phototactic response of the blind plus pinealectomized animals is not significantly different from the blind plus sham-pinealectomized group and cannot be considered as a positive response. Although these data clearly implicate the pineal organ of goldfish in the normal phototactic response of this species, they do not demonstrate a direct photoreceptive role of this organ. But they do indicate that the eyes and the pineal organ are both required for phototactic responses in goldfish.

Pinealectomy, Blinding, and Conditioning to Light and Sound

The preceding experiment suggests that the pineal organ is related to phototaxis but is unable to produce a behavioural response to light in the absence of the eyes. A second experiment was used to test this possibility. The various groups of
Figure 1. Distribution of goldfish in a light gradient following Blinding, Pinealectomy of both. Horizontal and vertical lines are means and 95% confidence intervals respectively calculated from 20 observations on ten fish per observation. (Overlap of confidence intervals taken as nonsignificance at the .05 level.)
<table>
<thead>
<tr>
<th>Group</th>
<th>Number in Light Half of Tank</th>
</tr>
</thead>
<tbody>
<tr>
<td>PINOXED</td>
<td>5</td>
</tr>
<tr>
<td>SHAM-PINOXED</td>
<td>4</td>
</tr>
<tr>
<td>NORMAL</td>
<td>3</td>
</tr>
<tr>
<td>BLIND</td>
<td>2</td>
</tr>
<tr>
<td>BLIND + SHAM-PINOXED</td>
<td>1</td>
</tr>
<tr>
<td>BLIND + PINOXED</td>
<td>0</td>
</tr>
</tbody>
</table>
goldfish listed in Table II were conditioned to escape an electrical shock by swimming over a barrier in response to a light or to a ringing door bell. The rationale behind these experiments was as follows. Animals which possessed both pineal and eyes would retain a negative phototactic response and more often escape the electrical shock by swimming away from the light and over the barrier than animals with eyes but without a pineal. Blind animals which possessed an intact pineal organ could be "motivated" by an electrical shock to respond to the light, thereby providing evidence for the photoreceptive ability of the pineal. By repeating the experiment using sound as the conditioned stimulus, it would be possible to judge whether the results from the light experiment were due to changes in response to light per se or resulted from a general alteration in sensitivity to all environmental stimuli.

Table II combines the results of these two experiments. An analysis of variance showed significant differences (Tukey's w-procedure, Steel and Torrie, 1960) of 7.12 for the light experiment and 3.92 for the sound experiment (P = .05). Using these values to make appropriate comparisons in Table II, it is apparent that eyed animals, as opposed to blind animals, showed a significantly greater response to light but that the reverse occurred when the conditioned stimulus was sound.

Pinealectomy did not affect the responsiveness of eyed or blind animals to sound, nor did it affect the responsiveness of blind animals to light. On the other hand, pinealectomy resulted in a significantly greater responsiveness to light when
TABLE II

Effect of Pinealectomy, Blinding, or both on the conditioning of goldfish to light and sound. The tabulated values represent the mean number of conditioned responses out of 50 trials.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Light</th>
<th>Sound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blind</td>
<td>Eyed</td>
</tr>
<tr>
<td>Pinealectomized</td>
<td>3.4</td>
<td>32.6</td>
</tr>
<tr>
<td>Sham-pinealectomized</td>
<td>3.8</td>
<td>24.2</td>
</tr>
<tr>
<td>Unoperated controls</td>
<td>4.4</td>
<td>19.8</td>
</tr>
</tbody>
</table>

*Significant differences of Tukey's w at the 0.05 level of significance were calculated as 7.12 for the light experiment and 3.92 for the sound experiment.
the pinealectomized animals retained their eyes. This result is diametrically opposed to the expected as these fish were previously found to have lost their negative phototactic response and should be less inclined to swim away from the light than the wholly intact animals which remain photo-negative. The inability of blind goldfish with a pineal to use light as a conditioned stimulus more often than blind goldfish without a pineal is taken as further evidence that the role of the photoreceptive function of the pineal organ depends upon the presence of normal vision.

The failure of pinealectomy to produce any changes in the responsiveness to sound indicates that the removal of the pineal gland does not influence responsiveness to all environmental stimuli.

Pinealectomy and Response to Electrical Stimulation

Pinealectomy did not influence the distance that goldfish moved when stimulated with the unconditioned stimulus from the preceding experiment (80 volts); that is, the differences noted in the conditioning experiment were the result of changes in response to the light and not the electrical shocks (Table III). But pinealectomized fish did have a lower minimum response voltage relative to the sham-pinealectomized and intact control groups, the differences being significant at the .05 level (Tukey's $W_{.05} = 2.68$). Although the implications of this finding are not immediately apparent, it does indicate a general increase in activity or excitability of the pinealectomized
TABLE III

Effect of pinealectomy on the response of goldfish to electrical stimulation.

Values given are the lowest voltages which will just cause a perceptable reaction or the units (2.5 cm) moved in response to a single shock of 80 volts. All values are averages of morning, noon and afternoon values taken from five separate fish tested on different days.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Minimum Effective Voltage</th>
<th>Distance Moved to 80 volts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pinealectomized</td>
<td>7.93</td>
<td>4.93</td>
</tr>
<tr>
<td>Sham-pinealectomized</td>
<td>10.79</td>
<td>4.11</td>
</tr>
<tr>
<td>Intact controls</td>
<td>10.23</td>
<td>4.29</td>
</tr>
</tbody>
</table>

*Significant difference of Tukey's w at 0.05 level of significance was calculated as 2.68 for minimum effective voltage.
group. This possibility will be considered in the discussion.

Pinealectomy, Blinding, and Swimming Activity

The effect of pinealectomy, blinding, or both, on the mean number of total lines crossed by three goldfish during three 10 minute intervals is presented in Figure 2. The data from which these means were calculated were subjected to a two way analysis of variance from which a significant difference of 93.4 was obtained (Tukey's W procedure, P < .01). Table IV presents data to show diurnal variation in swimming activity and the effect of various treatments.

The blind groups showed significantly more swimming activity than the eyed groups. Within the eyed groups, the pinealectomized fish were more active than either the sham-pinealectomized or the intact control group; the reverse was true among the eyeless groups. The differences mentioned are significant at the .01 level.

A marked diurnal rhythm in activity was found (Table IV). Among the eyed groups, activity dropped from a peak level in the morning to a low level at noon and then rose slightly during the afternoon. The blind groups displayed peak swimming activity at noon. Pinealectomy did not appreciably affect the activity cycles.

Effect of Pinealectomy, Blinding, or Both on Brain Serotonin Levels

Pinealectomy produced no appreciable alteration in whole
Figure 2. Swimming activity of normal goldfish or following pinealectomy, blinding, or both. Mean number of lines crossed in a circular channel during three 10 minute intervals (AM, noon, and PM). The top of the bars represent the mean number of lines crossed by five groups of three goldfish from each of the treatments. Pinealectomized, P; sham-pinealectomized, S; unoperated controls, C; blind and pinealectomized, BP; blind and sham-pinealectomized, BS; and blind, B.
activity units per 100

P
S
C
BP
BS
B
**TABLE IV**

Swimming activity of goldfish showing diurnal rhythm and effect of pinealectomy, blinding, or both.

Activity units are average number of lines crossed by three goldfish in a circular channel during a 10 minute period. Values are averages of five 10 minute intervals taken on different days using different groups of three fish.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average for 5 days</th>
<th>Total Units for 5 groups of 3 fish</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>am</td>
<td>noon</td>
</tr>
<tr>
<td>Pinealectomized</td>
<td>131.8</td>
<td>31.0</td>
</tr>
<tr>
<td>Sham-pinealectomized</td>
<td>66.8</td>
<td>11.6</td>
</tr>
<tr>
<td>Intact controls</td>
<td>69.8</td>
<td>11.0</td>
</tr>
<tr>
<td>Blind</td>
<td>191.3</td>
<td>233.4</td>
</tr>
<tr>
<td>Blind plus pinealectomized</td>
<td>112.8</td>
<td>157.0</td>
</tr>
<tr>
<td>Blind plus sham-pinealectomized</td>
<td>191.6</td>
<td>228.8</td>
</tr>
</tbody>
</table>
brain serotonin content (Table V). But blinding resulted in a significant decrease in brain serotonin when compared to the levels found in the eyed groups (Tukeys W _{0.05} = 0.09). When the mean serotonin levels for each group of goldfish were compared to the corresponding mean swimming activities (Figure 3), a negative correlation was obtained (r_s = 0.83) which was just significant at the .05 level (Spearmans Coefficient of Rank Correlation, r_s, Siegal, 1956). However, this relationship must be viewed with caution since a correlation provides evidence only about the joint relationship between two variables and does not establish cause and effect. Nevertheless, this finding does not refute the possibility of a functional relationship between serotonin levels and swimming activity.
TABLE V

Effect of pinealectomy, blinding, or both on the serotonin content in goldfish brains. Each tabulated value represents a mean of five determinations on five fish.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Eyed Groups</th>
<th>Blind Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pinealectomized</td>
<td>0.36</td>
<td>0.24</td>
</tr>
<tr>
<td>Sham-pinealectomized</td>
<td>0.34</td>
<td>0.21</td>
</tr>
<tr>
<td>Control</td>
<td>0.44</td>
<td>0.19</td>
</tr>
</tbody>
</table>

*Tukey's w.05 = 0.09.
Figure 3. Relationship between mean total swimming activity and mean brain serotonin in various experimental groups of goldfish. Pinealectomized, P; sham-pinealectomized, SP; unoperated controls, C; blind and pinealectomized, BP; blind and sham-pinealectomized, BS; and blind, B.
Evidence for a photoreceptive role of the fish pineal organ is derived from direct neurophysiological studies (Dodt, 1963; Morita, 1966), morphological studies (Rivas, 1953), and indirect investigations involving the histological (Holmgren, 1959a; Eakin, 1963; Rüdeberg, 1968a, b) and ultrastructural (Kelly, 1962; Rüdeberg, 1966) appearance of this organ. Valuable information concerning its function has been obtained by tests involving color changes (Young, 1935; Breder and Rasquin, 1950; Hoar, 1955; and Schonherr, 1955), and behavioural responses (Breder and Rasquin, 1947; Hoar, 1955; and Pang, 1965). Despite the disparate experimental approaches, all of these studies indicate a direct photosensory role of the fish pineal organ.

The present investigation shows that the pineal organ of goldfish is involved in phototaxis and responsiveness to light stimuli but offers no evidence that the pineal organ has photoreceptive abilities. On the other hand, from previous reports, there is no unequivocal evidence for efferent nerve fibers to the fish pineal organ, light has been found to affect the histology of the pineal organ, and illumination of the pineal results in altered nervous activity within this organ. So in spite of the results of this experiment, the evidence is strongly in favour of the hypothesis that the pineal organ of fishes is photosensitive. If this is true, how is it that the pineal organ of goldfish is unable to elicit a response by the animal
to illumination when the eyes are absent?

Among fish with intact lateral vision, pinealectomy results in a loss of phototaxis, and an apparent increase in the responsiveness to light when light is used as a conditioned stimulus. However, fish without eyes but with a pineal show the same responses to illumination as blind fish without a pineal. A similar situation was reported earlier by Breder and Rasquin (1947) who found that in the blind Mexican cave fish *Anoptichthys* phototaxis depended upon the presence of the remnant optic cysts although the sign of phototaxis was related to the pineal organ. These findings together suggest that the role of the pineal organ in photoreception is to alter the responses elicited by the photic information received by the eyes so that the normal phototactic response depends upon the function of the eyes as well as the pineal organ.

To understand more fully how the pineal can be a photoreceptor and still be unable to produce behavioural responses to illumination it is necessary to remember two of the different effects of light. The first effect is to produce the complex physiological-psychological experience of vision to which an animal may be behaviourally responsive. The second effect is the stimulatory effect on autonomic reflexes which the animal may or may not experience and over which the animal has little or no control. If this second effect, or autonomic reflex, is to modulate the response to the first effect, or experience of visual stimuli, then the integrated response resulting from both effects will not occur in the absence of either effect. In other
words, if the photoreceptive ability of the pineal organ of goldfish is associated with an autonomic reflex which modulates the response to visual stimuli received via the eyes, then removal of either the pineal or the eyes will result in a loss of that response. Thus, with regard to phototaxis, the pineal organ of the goldfish is associated with the reception of photic stimuli which the animal does not experience, and the information so received produces no effect in the absence of the lateral eyes.

The data from the conditioning experiments supports this conclusion. Blind animals with a pineal organ could not be effectively trained to use light as a conditioned stimulus to avoid an electrical shock even though all of the blind groups could be trained to respond to sound. Conversely, animals with eyes but without a pineal showed a greater responsiveness to the light and avoided the shock more often than animals with both pineal organ and eyes.

As mentioned in the introduction to this section, phototaxis depends upon the possession of a preference for a particular degree of brightness but the actual response is displayed only when the fish has a readiness to move from one condition of brightness to another. Pinealectomy eliminated the negative phototactic response of goldfish but also resulted in an apparent increased responsiveness to light as evidenced by the increased number of conditioned responses in which the animal swam from the light half of the tank to the dark half. This suggests that the pineal organ is associated with the
"willingness" of the fish to change the condition of brightness under which the fish finds itself rather than the preference for any particular degree of brightness.

The differences in response to light following pinealectomy were not due to changes in sensitivity to the unconditioned stimulus. The distance that pinealectomized goldfish swam when stimulated with the unconditioned stimulus was the same as the distance swam by wholly intact and sham operated fish. Further, although pinealectomy resulted in a reduced minimum response voltage and an increase in swimming activity, it had no effect on the number of conditioned responses elicited by the ringing doorbell. From these data it was concluded that the sensory role of the pineal organ in goldfish is specifically related to photic stimuli.

The effects of pinealectomy and blinding on the phototactic response of various fishes have been explored previously. Hoar (1955) reported the presence of phototaxis in blinded salmon smolts with intact pineals, and therefore differed in his conclusions from those reported here. The difference in the results of these experiments may be explained by the age of the animals used, or in the intensity of light employed. Hoar (1955) suggests that developmental differences in the degree of negative phototaxis shown by young sockeye salmon is related to the degree of pineal development. Indeed, the pineal organ of fishes becomes increasingly invaginated with age (Hoar, 1955), and undergoes a decrease in sensory cell number with continued development (Hafeez and Ford, 1967) together with a marked
The degeneration of nervous elements (Kappers, 1965). Thus, the pineal body of fishes may change from the only photosensory structure involved in phototaxis in young fish to a subsidiary photosensory structure in the older fish where it can no longer autonomously produce a phototactic response. On the other hand, Hoar (1955) performed his experiments under natural light where the light intensity was many times greater than that employed in the present study. Previously, von Frisch (1911a,b) and Young (1935) suggested a general light sensitivity of the diencephalic roof and since the smolts used by Hoar (1955) had thin skulls, the phototaxis demonstrated by his blind fish could have resulted from the effect of high light intensity on the brain itself. Nevertheless, the possibility of species differences in pineal function can not be disregarded.

These data, then, lead to the conclusion that the sensory role of the pineal organ of the adult goldfish is specifically related to photic stimuli. Further, that this role depends upon, and is subsidiary to, intact lateral vision so that with regards to distributional responses to light, the eyes and the pineal organ function together. When the results from the literature are considered as well, the following hypothesis emerges. In young fish, the pineal organ autonomously regulates the phototactic response by operating as the receptor site for stimuli governing the persistence tendency of Janzen (1933). In older animals, the pineal retains control over the persistence tendency but the lateral eyes acquire the function of controlling the brightness preference. The importance of the
photosensory role of the fish pineal organ decreases with advancing age. This decrease parallels the loss of the pineal photoreceptive ability seen between fish and mammals and might therefore represent another example of ontogeny recapitulating phylogeny.

No previous reports have appeared on the effect of the pineal gland on the general activity of fish. This is surprising in view of the thyroidal hypertrophy and suggested increase in gonadal endocrine activity seen in young *Lebistes* (Pflugfelder, 1953, 1954) and the hyperthyroidism of goldfish (Pflugfelder, 1964) following pinealectomy. Hoar et al. (1955) have shown that thyroxine and gonadal steroids when administered exogenously, result in increased locomotory activity in goldfish as well as a decrease in the strength of electrical stimuli required to cause goldfish to move a standard distance. Similar results were obtained in this study following pinealectomy of animals with intact lateral vision. The possibility of such endocrine effects will be discussed in the General Discussion at the end of the thesis.

Another possible reason for the changes noted in swimming activity following pinealectomy was investigated. Serotonin is found in high concentrations in the pineal gland (Quay, 1964, 1965) and can cause marked sedative effects on the level of activity (Weisman, 1967). Thus, if the pineal organ of fish is a major source of brain serotonin, or can increase brain serotonin levels, then removal of the pineal organ might result in a decreased brain serotonin level, increased excitability, and
increased swimming activity of the fish. The results obtained from this study do not support this hypothesis (Table IV and Fig. 2). Pinealectomy did not alter the level of whole brain serotonin. However, as the results reported herein are for whole brains and as serotonin is known to be unevenly distributed between the anatomical regions of the brain (Quay and Wilhoft, 1964), the possibility that pinealectomy caused localized changes in serotonin levels can not be ruled out. At any rate, Figure 3 suggests a negative relationship between the level of brain serotonin and the level of swimming activity.

At present, there is insufficient evidence to explain how the fish pineal organ functions in photoreception. There is, however, ample evidence that the pineal organ of the goldfish is associated with behavioural responses to light. Further, the effects of pinealectomy on the swimming activity and minimum effective voltages to cause a response in goldfish suggests a second function of the pineal organ related to neural activity. Whether these two functions are separate, or whether one is the result of the other is not known. Nevertheless, the pineal organ of the goldfish does have subtle but basic and important functions.
SECTION II

DEMONSTRATION AND EFFECT OF MELATONIN IN FISH

INTRODUCTION

Though the question has been raised (Quay, 1965; Oksche' and Kirschstein, 1967; Rüdeberg, 1968), it is not known whether melatonin (N-acetyl-5-methoxytryptamine, Learner et al., 1958) which has been found present in the pineal organs of mammals (Wurtman and Axelrod, 1965), birds (Ralph et al., 1967) and amphibians (Charlton, 1964; Van de Veerdonk, 1967) is present in the pineal organ of fishes. Melatonin often (Wurtman et al., 1963; Chu et al., 1964; Adams et al., 1965; Fraschini et al., 1968) though not consistently (Reiter, 1967) exerts antigonadal effects in mammals similar to those of bovine pineal extracts (Kitay and Altschule, 1954; Wurtman et al., 1959; Mayer et al., 1961) and neutralizes some of the effects of pinealectomy (Motta et al., 1967; Fraschini et al., 1968). These findings support the speculation that melatonin is a pineal hormone in mammals (Wurtman and Axelrod, 1965), and that the pineal organ of mammals is a functional endocrine organ (Thiéblot, 1965).

A similar function of the pineal organ in fishes has been suggested. Krochert (1936a,b) found that the growth rate and the appearance of secondary sexual characteristics were retarded in young guppies Lebistes fed dessicated bull pineal glands. Pflugfelder (1953, 1954), also working with guppies, found that pinealectomy induced a slight acceleration in the appearance
of secondary sexual characteristics in young males. Recently, Quay (1965b) localized the enzyme necessary for melatonin synthesis within the pineal organ of fishes. Together, these findings suggest that the pineal organs of mammals and fishes are functionally similar with regard to synthesis of melatonin and its ensuing inhibition on the reproductive system. The acceptance or rejection of this hypothesis depends to a large extent upon the demonstration of a pineal melatonin component in the fishes and the establishment of some effect of melatonin on the reproductive or endocrine system of fish. Further, evidence for the participation of melatonin in the reproductive process of fish might be acquired if a relationship between pineal melatonin stores and the state of reproductive maturity could be demonstrated under natural conditions.

Quay (1965b) also found a retinal component of the methylating enzyme, hydroxy-indole-O-methyl transferase (HIOMT), the enzyme which converts N-acetyl-serotonin to melatonin, in fishes, and suggested the possibility of compensatory increases in retinal melatonin production following decreased production in the pineal. For this reason, and for the purpose of interpreting experiments involving pinealectomy of goldfish, the relationship between pineal and retinal melatonin levels was examined.

To this end, melatonin, and its precursor serotonin, were examined qualitatively and quantitatively in the pineal organ and retinas of the large Pacific salmon, the chinook or spring. This animal was chosen for the large size of its pineal organ plus the fact that this species exhibits a marked and prolonged
reproductive cycle which enabled immature and mature salmon of similar sizes to be compared. Goldfish were used to study the effect of exogenous melatonin on the reproductive system and selected endocrines. These animals are easily acquired, maintenance is minimal, and they are hardy enough to withstand daily injections.

This section reports on qualitative and quantitative studies for retinal and pineal melatonin in mature as well as immature salmon and attempts to relate the findings to the reproductive cycle of this species. The effects of intraperitoneal injections of melatonin on the goldfish gonads, interrenal nuclear diameter, thyroidal tissue and pituitary gonadotrophs are described and the possible mode of action is discussed.
Melatonin Injection Studies

Goldfish, common variety (7.5 - 10 cm size class), were procured from the Goldfish Supply Company, Stoufville, Ontario, and arrived at the laboratory on 21 December, 1967. They were placed in a holding trough of approximately 400 liter capacity containing dechlorinated water (pH 6.4 - 6.9). The light regimen within the holding tank was maintained at 8 hours of light alternating with 16 hours of darkness and the temperature of the water ranged from 11 C to 12 C. All fish, both stock and experimentals, were fed Clark's New Age dry fish food once daily.

On the 20th of February, 1968, 48 fish were transferred to each of two experimental tanks set up in a controlled environment room maintained at 10 C. The tanks were filled with 300 liters of water containing equal parts of NaCl and CaCl_2 to a final concentration of 0.2%; Vancouver city water is low in dissolved solids and the salts also prevented Saprolegnia infection of the fish. To further prevent this infection, one drop of 1% aqueous solution of Malachite Green was added to each 100 liters of water. The water was held at 20 C ± 0.05 C with suitable thermostats, relays, and heating coils. Photoperiods within the tanks were achieved by fixing three 40 watt incandescent bulbs to the roof of the light tight covers fitted to each tank. The lights were activated through a timing device. This system yielded 25 foot candles of illumination at the
surface of the water while the lights were on and eliminated perceptible light when the lights were off. The light regimens within the separate tanks were: 1 hour of light alternating with 23 hours of darkness (abbreviated 1L); and 8 hours of light alternating with 16 hours of darkness (abbreviated 8L). After 35 days, 12 fish were removed from each tank and killed. Body weights and gonad weights were taken and the gonosomatic index (GSI = GONAD WEIGHT/BODY WEIGHT X 100) was calculated for each fish. The remaining 36 fish in each tank were then divided into groups of 12 fish and each group was placed in a separate floating, nylon-screen basket enclosing 12,000 cm$^3$ of water. The photoperiod within both tanks was set at 16 hours of light alternating with 8 hours of darkness (abbreviated 16L). On the same day, the fish in one basket from each tank were injected with melatonin, the fish in another basket were injected with the aqueous carrier only, while the fish in the remaining basket were left as untreated controls. The fish were injected once a day for 50 days. All fish were then killed, GSI's were calculated for each fish and the gonads, head kidneys containing the interrenal tissue, thyroidal tissue from the throat region and whole heads were fixed for histological examination. There were no differences between the gonad size of animals exposed to 1L and 8L prior to injection, or between fish that had received similar treatment under 16L. Therefore, the data from the two tanks were pooled prior to statistical analysis.
A. **Injection of Melatonin**

Each fish received 20 μg of melatonin (Mann Research Laboratories, N.Y.) per day dissolved in 0.25 ml of freshwater teleost Ringer's solution (Hoar and Hickman, 1967). The solvent injected animals received only the Ringer's solution. The daily injection alternated from the left to the right side of the animal.

For the injections, a short, #30 gauge needle fitted to a 0.25 ml tuberculin syringe was used. Fish were not anaesthetized, but were held in a bare wet hand. The needle was inserted into the abdominal cavity in an area 2 mm below the lateral line and 5 mm forward of an imaginary perpendicular passing through the vent. The needle was directed forward and slightly ventrad and the point of the needle was kept near to the body wall to prevent puncture of the visceral organs. Between the daily series of injections, the needle and syringe were stored in 99% ethanol. The dose of 20 μg of melatonin per day approximates the dose used in mammals (Adams et al., 1965; Panda and Turner, 1968) were the mammals of equivalent size to the fish employed in this study.

B. **Histological Procedures**

Gonads and head kidneys containing the interrenal tissue were fixed in Bouin's picric acid-formal-acetic acid solution (75:25:5). Thyroid tissue from the region of the throat and whole heads were fixed in modified Bouin's solution with the acetic acid replaced by formic acid to decalcify any contained
bone. Pituitary glands were dissected from the heads following
decalciﬁcation. All tissues were embedded in parafﬁn and
sectioned at 6 μ. The tissues, with the exception of the pitui-
tary, were stained with haemotoxylin and eosin. The pituitaries
were stained with Alcian blue-periodic acid Schiff (PAS)-orange
G.

C. Analysis of Histological Results

Quantifiable measurements were not made on the histological
appearance of the testis or ovaries. General observations based
on ﬁve animals of each sex from each experimental condition
were recorded to ascertain the state of maturation of the gonads.

Apparent thyroidal activity was based on the assigning of
integers from one to ﬁve to represent the relationship between
cell height and nuclear diameter where 1 indicates a nuclear
diameter greater than the average cell height; 2, a nuclear dia-
meter equal to the average cell height; 3, a cell height greater
than, but less than twice, the nuclear diameter; 4, a cell height
between two and three times the nuclear diameter; and 5, a cell
height greater than three times the nuclear diameter. Twenty-
ﬁve recordings were made from each ﬁsh, ﬁve from every tenth
section. The recordings from a single ﬁsh were averaged to
produce a single value for that ﬁsh. Five melatonin injected
and ﬁve solvent injected ﬁsh were analyzed in this way.

Interrenal nuclear diameters were measured with an ocular
micrometer. When the nuclei were not round, the sum of the long
and short axis was halved to provide a single value. Twenty-
five nuclei from each fish were measured; five measurements were made on every fifth section. The 25 nuclear diameters from each fish were averaged to obtain a single value for each fish. Five melatonin injected and five solvent injected fish were compared. Ocular units were converted to microns prior to statistical testing.

The cell diameters of pituitary gonadotrophs were measured in the same way as the interrenal nuclei but the statistics were performed directly on the ocular units. Ten gonadotrophs were measured in each of five melatonin injected and five solvent injected animals. As there were no significant differences between individual fish within the same group, values from one fish were not averaged but were considered independent observations. For comparison purposes, the sample size was considered to be 50.

D. Statistical Methods

One way analyses of variance were performed on the male and female GSI data separately. Individual differences between means were tested with the least significant difference (lsd) procedure as modified for unequal replication (Steel and Torrie, 1960). But t-tests were used to compare differences in thyroidal cell height, gonadotroph diameter and interrenal nuclear diameters from melatonin and solvent injected animals.
Qualitative and Quantitative Estimation of Melatonin and Serotonin

A. General

During the course of this study it became evident that slight changes in procedure or chemicals resulted in marked alterations in the extraction and fluorometry of indoles. For this reason, the source of important chemicals and a brief resume of some standard procedures are provided.

All water used was deionized by passage through a Bantam Model BD-1 demineralizer (Barnstead Still and Sterilizer Co., Boston, Mass.) and distilled in an all glass still (Corning, Model AG 1A). Ascorbic acid was obtained from the Nutritional Biochemical Corp., Cleveland, Ohio, EDTA-K₂ and EDTA-Na₂ from the Cambridge Chemical Products Inc., Detroit, Mich., and the boric acid was purchased from Allied Chemical and Dye Corp. of New York. Reagent grade NaCl, NaOH, and KOH were procured from the Nichols Chemical Co., of Montreal, P.Q. Heptane was bought from the Eastman Organic Co., Rochester N.Y. Diethyl-ether, HCl, KCl, di-potassium hydrogen orthophosphate, and cyclohexane were obtained from the British Drug Houses, Poole England, and the n-butanol and p-cymene were bought from Fisher Scientific Co., Fairlawn, N.J. All indole standards were purchased from the Mann Research Laboratories, N.Y., and were thin-layer chromatographically pure (Mann Assayed).

Organic solvents were distilled twice prior to use. In addition, the p-cymene was washed twice in each of 1N NaOH, 1N HCl, and distilled water prior to distillation. The initial
p-cymene distillate was discarded; the subsequent fraction (176C-178C) was collected for use (Quay and Baker, 1965).

Concentrated standard solutions of the indoles were prepared by dissolving the indole in a small amount of ethanol (when required) and diluting with sufficient 0.1N HCl or 0.1N HCl, 0.5% ascorbic acid to bring the concentration of indole to 250 mg per ml of solution. Working standards were prepared and checked daily.

All glassware was washed with Calgonite as this type of soap does not leave fluorescent residues (Udenfriend, 1962). When necessary, the cuvette used during fluorometry was washed by boiling in 50% HNO₃ after the method of Udenfriend (1962).

The buffer used for serotonin extractions was made according to Wise (1967b). Crystalline di-potassium hydrogen orthophosphate (252.2 gm) was dissolved in 400 ml of water. The pH was adjusted to 10.00 with concentrated KOH solution and the resulting solution was saturated with KCl. The volume of the buffer was then increased to 500 ml with water. A small amount of KCl was left at the bottom of the buffer which was stored at room temperature. Fresh buffer was prepared every two weeks. For melatonin extraction, the borate buffer (pH 10.00) used by Quay (1963) was employed. It was prepared by dissolving 59.6 gm of boric acid and 41.7 gm of NaOH in two liters of water. The borate buffer was saturated with NaCl and p-cymene prior to use.
B. **Source of Biological Material**

Sexually mature salmon were obtained from fresh water at the Washington State Department of Fisheries' Little White and Spring Creek salmon hatcheries located approximately 100 miles above the mouth of the Columbia River. Only reproductively mature salmon were used: the criterion for maturity was the actual use of the animal as a source of roe or milt. The immature salmon were taken by commercial trawlers in the Pacific Ocean off the coast of Vancouver Island. No attempt was made to determine if the mature and immature salmon represented a similar spawning population, nor was the sex of the animals recorded.

Fish were killed by cutting a core of tissue, 2.5 cm in diameter, from the head in the region of the brain as described by Tsuyuki *et al.* (1964). Pineal organs were immediately dissected from the core of brain tissue and were stored in polyethylene bottles over solid CO$_2$. Whole eyes were stored in the same manner following removal from the orbits. Retinas were removed as required from partially frozen eyes.

C. **Preparation of Extracts for TCL**

Pineals or retinas were pooled separately to yield 2 gm and 5 gm respectively (approximately 7 pineals or 12 retinas). The tissue was homogenized in 15 ml of ice-cold 0.1M anhydrous sodium phosphate (monobasic). The homogenate was extracted twice with two volumes of ice-cold ethyl acetate and the extracts were pooled. Anhydrous sodium sulphate (30 gm) was added to the pooled extracts to remove excess water and the mixture
was allowed to stand one hour. This mixture was then filtered through a medium cintered glass filter to remove the sodium sulphate and any pieces of tissue. Finally, both the filter and the sodium sulphate were washed with several ml of ethyl acetate which were then added to the previous filtrate. The filtrate was evaporated to dryness by flash evaporation at 20°C under an atmosphere of nitrogen. The residue was taken up in 2 ml of a mixture consisting of absolute ethanol and 0.1N HCl (4:1), and the resulting solution was evaporated under a reduced nitrogen atmosphere to 0.5 ml. Test indole solutions were prepared by dissolving commercial indoles directly in the ethanol-0.1N HCl solution. The extraction procedure was checked by extracting water, serotonin and melatonin standards. Water extracts produced no spots when run chromatographically. Both the serotonin and the melatonin standards were found to be chromatographically pure; that is, each indole yielded only one spot following development and detection of indoles. Recovery of serotonin and melatonin from standard solutions was excellent and approached 100%. Extracts were chromatographed on the same day as prepared.

D. **Thin Layer Chromatography**

Chromatographic separations were performed on glass plates (20 cm x 20 cm) coated on one side to a depth of 0.25 mm with the absorbant Silica Gel G (Merck). Plates were air dried and stored in a desiccator over anhydrous calcium chloride. Prior to use the plates were sprayed with a solution of 0.5% ascorbic
acid in absolute methanol to enhance the survival and recovery of the 5-hydroxy and 5-methoxy compounds (Quay and Bagnara, 1964).

Ascending, two dimensional development was used for the separation of the indoles. The developing chambers were of the standard rectangular trough type with the inside of three walls covered with a layer of thick filter paper to enhance equilibration of the solvent systems. The chambers were allowed to equilibrate for a minimum of two hours following addition of 100 ml of the solvent system. The following solvent systems proved to be the most useful for this study (Stahl, 1965):

- **Alkaline system:** methyl acetate-isopropanol-$25\% \text{NH}_3$ (45:35:20)
- **Acidic system:** chloroform-96\% acetic acid (95:5)

For the actual separations, 0.1 ml of the tissue extract was spotted on the plate at a point 2.5 cm equidistant from two sides. Along both of these sides and 2.5 cm from the edge of the plate, an identical series of standards was spotted; each spot was 2 cm distant from another. Sufficient standard extract was applied to each spot to bring the total amount of indole spotted to 0.1 ug. The plate was then placed in the chamber containing the alkaline system and the solvent front was allowed to rise to a distance of 10 cm above the original line of spots. Following development in the first direction, excess \text{NH}_3 was removed by placing the plate over concentrated \text{H}_2\text{SO}_4 in a desiccator, evacuating to 15-20 mm of Hg, filling the
desiccator with N₂, reevacuating (this was done several times), and then allowing the plate to stand in the reduced N₂ atmosphere over H₂SO₄ for one hour. The plate was then removed from the desiccator, rotated 90° to the direction of the first development, and developed in the acidic system until the solvent front had risen to a point 10 cm above the spots along the second side.

E. **Demonstration and Identification of Indoles**

For the detection and identification of the various indoles, the following reactions (Stahl, 1965) were employed and the Rf values (Rf = distance of spot from starting point (cm)/height of solvent front above starting point x 100) obtained in both solvent systems were compared with those of standard serotonin and melatonin extracts run at the same time on the same plate.

**General Detection of Simple Indoles**  The plates were sprayed with a freshly prepared mixture consisting of 10 ml of formaldehyde (35%, aqueous), 10 ml of 25% HCl, and 20 ml of absolute ethanol. The plate was then exposed to the vapours of aqua regia and heated for five minutes at 100 C. This reaction (Prochazka reaction) gives only yellow reaction products which fluoresce strongly in a long wave ultraviolet light. Although this is an extremely sensitive technique for demonstrating indoles, and is useful for the purpose of calculating Rf values of indoles present in low concentrations, it is not specific in its color reactions.

**Detection of Serotonin**  The plate was sprayed with
ninhydrin (0.25%) in a solution of acetone containing 10% of acetic acid. This is an extremely sensitive test for serotonin (Jepson and Stevens, 1953) but also reacted with other indole compounds. However, serotonin yields a blue-green fluorescence when the plate is heated for two or three minutes at 90 C-100 C.

Detection of Melatonin

The most specific of the techniques for the identification of simple indole derivatives (Stahl, 1965) and one which is particularly useful for demonstrating melatonin is the van Urk's reaction. The plate is sprayed copiously (approximately 10 ml for a 20 cm x 20 cm plate) immediately following development with a reagent (van Urk's) prepared by dissolving 1 gm of 4-dimethylaminobenzaldehyde in 50 ml of HCl and adding 50 ml of absolute ethanol to the resulting solution. After the plate was thoroughly wetted with van Urk's reagent, it was exposed briefly to the vapours of aqua regia. Mélatonin appears as a blue spot almost immediately.

Although no single technique employed for the demonstration of the indoles was specific enough to establish the presence of serotonin or melatonin, a combination of the two Rf values and the color reactions with the different techniques of detection enabled the accurate identification of these indoles. Though chromatography permitted estimates to be made about the relative quantities of serotonin and melatonin in different extracts, it did not yield accurate measures of concentration.
F. Fluorometric Techniques

The method employed for the fluorometric analysis of serotonin was described in Section I and will not be repeated here. Melatonin levels were measured fluorometrically following extraction with p-cymene from an alkaline solution as described by Quay (1963) and Quay and Baker (1965). Reproducibility, extraction efficiency and specificity obtained in the melatonin determinations were estimated in the same manner as described for serotonin (Section I) with the exception that 5-hydroxytryptophane was replaced by 5-methoxytryptamine. Reproducibility was excellent, the recorded melatonin levels from the same sample tested on different days varied within a few percentage points. Recovery of added melatonin ranged between 75% and 89%. For this reason, melatonin levels in tissue extracts were calculated from a standard curve obtained by extracting melatonin standards of different concentrations. Because extraction of 5-methoxytryptamine standards by the melatonin extraction procedure was found to yield a 6% recoverability, the specificity of the technique was judged inaccurate for establishing the presence of melatonin. However, when melatonin was present in reasonably high quantities (100 ng per sample), the technique yielded precise measures of melatonin concentrations. Using these techniques, the levels of serotonin and melatonin were measured in the pineal organs of mature and immature salmon and the level of serotonin only was measured in the retinas of the same animals.
RESULTS

Demonstration of Serotonin and Melatonin in Fish

Melatonin was found in the pineals of both immature and mature salmon but could not be found in the retinal tissue. Serotonin was found in the pineal and the retinas of immature and mature salmon.

A spot was found on the chromatoplates following TLC of either retinal or pineal extracts with Rf values of 64 and 2 in the alkaline and acidic systems respectively. The Rf value of 64 differs by one point (65) from that reported by Stahl (1965) for serotonin developed in the same manner. Further, both Rf values were identical to those obtained from serotonin standards developed simultaneously with the tissue extracts, and differed from other indoles tested. By way of identification, this spot turned grey with van Urk's reagent, yellow with Prochazka reagent, and fluoresced blue-green under ultraviolet radiation following treatment with the ninhydrin reagent. Since all of these reactions are characteristic of serotonin extracts, the spot was identified as serotonin.

The relative concentrations of serotonin in the pineal tissue appeared to be four or five times that seen in an equivalent amount of retinal tissue. No differences in the retinal or pineal serotonin levels were found between mature and immature salmon.

The decision for the presence of melatonin in the pineal tissue was based on the fact that the pineal extracts, when
chromatographed, yielded a spot with Rf values of 85 in the alkaline system and 16 in the acidic system, and that both of these values coincided exactly with the Rf values obtained from extracted standard melatonin run at the same time. No other indole tested had the same two Rf values. Further, when the plate was sprayed with van Urk's reagent, the spot turned a blue color which could be intensified by exposure to aqua regia vapours. Although other indoles produced the same reaction, the blue color did not appear as quickly as when melatonin was the reacting indole. When estimated by this technique, the relative amount of melatonin in the pineal of immature salmon appeared to be three or four times that seen in the pineals of mature salmon. Melatonin was not found in other parts of the brain, the blood, or muscle tissue.

Serotonin and Melatonin Levels in Immature and Mature Salmon

Pineal and retinal levels of serotonin, and pineal levels of melatonin, in mature and immature salmon, are summarized in Table VI. Melatonin was not identified in the retinal tissue by chromatography and was therefore not measured fluorometrically in this tissue.

The tabulated values in Table VI represent mean levels of the indoles calculated from five pooled samples of pineal or retinal tissue. Although the level of serotonin found in the pineal glands was approximately four times as great as that found in the retinas, there were no differences in the level of serotonin between mature and immature salmon in either tissue.
TABLE VI

Serotonin and melatonin content of mature and immature salmon pineals and retinal tissue.*

<table>
<thead>
<tr>
<th>Indole</th>
<th>Pineals</th>
<th>Retinas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immature</td>
<td>Mature</td>
</tr>
<tr>
<td>Serotonin</td>
<td>2.08±0.12</td>
<td>2.21±0.08</td>
</tr>
<tr>
<td>Melatonin</td>
<td>4.49±0.72</td>
<td>0.68±0.08</td>
</tr>
</tbody>
</table>

*The figures are means ± S.E.M. based on five pooled extractions each; the tabulated values are in ug/g of wet tissue.

**One tailed test and therefore the mean level of pineal melatonin in the immature salmon is significantly (P<.005) greater than the mean level of melatonin in the mature salmon.
These results corroborate those reported from the TLC studies. On the other hand, as suggested by the qualitative studies, the level of melatonin found in the pineal glands of immature salmon was significantly ($P < 0.005$) higher than that found in the pineal glands of mature salmon. The approximate amount of melatonin in single pineal glands was 180 ng in the mature fish as opposed to 1200 ng in the immature fish. The similarity in the relationships between the amount of serotonin and melatonin in the pineals of immature and mature salmon as estimated by thin layer chromatography and fluorometry was taken as further evidence that the indoles were correctly identified.

Effects of Melatonin Injection

The effects of melatonin injection on the GSI of male and female goldfish are summarized in Table VII. The tabulated values are mean GSI's of male and female goldfish prior to melatonin injection and held under short photoperiod (1L or 8L) and after fifty days of exposure to long photoperiod (16L) with or without melatonin or solvent injections. Analysis of variance were run on the male and female data separately (Table VIII). The least significant differences for single comparisons were calculated from the appropriate analysis of variance and were used to test the significance of differences between mean GSI values from the different experimental groups.

Melatonin inhibited the increase in the GSI of male and female goldfish exposed to long photoperiod. Male and female goldfish which were not injected or had received injections of
### TABLE VII

Effect of melatonin injection on the GSI of male and female goldfish following exposure to long photoperiod (16L).

<table>
<thead>
<tr>
<th>Sex</th>
<th>Initial Controls</th>
<th>Final Controls</th>
<th>Solvent Injected</th>
<th>Melatonin Injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>0.37(10)</td>
<td>1.53*(14)</td>
<td>1.59*(8)</td>
<td>0.50(12)</td>
</tr>
<tr>
<td>Female</td>
<td>1.37(13)</td>
<td>4.31*(10)</td>
<td>4.90*(16)</td>
<td>1.64(12)</td>
</tr>
</tbody>
</table>

Values are means (Number of observations).

1Pooled values of animals held under 1L and 8L for 35 days but before exposure to 16L.

2After 50 days of 16L.

*Represents a significant increase from mean GSI of initial controls (P<.05).

**Represents a significantly larger GSI when compared to the melatonin injected groups (P<.05).
### TABLE VIII
Analysis of variance of male and female GSI data before and after 50 days of melatonin or solvent injection.

#### Males:

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>( F_{0.01 (3,40)} = 4.31 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>13.72</td>
<td>3</td>
<td>4.57</td>
<td>6.35</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>28.78</td>
<td>40</td>
<td>0.72</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Therefore treatment is significant

#### Females:

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>( F_{0.01 (3,47)} = 4.08 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>131.21</td>
<td>3</td>
<td>43.74</td>
<td>4.49</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>457.46</td>
<td>47</td>
<td>9.73</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Therefore treatment is significant
the solvent only had significantly (P < .05) larger gonads after 50 days exposure to long photoperiod than the male or female fish sampled prior to exposure to long photoperiod. The melatonin injected animals did not show a significant increase in the size of their GSI's during the 50 days of exposure to long photoperiod. Failure to find a significant difference between the uninjected female controls and the melatonin injected females (Table VII), plus the fact that both the male and female melatonin injected groups showed slight increases in their GSI values during the period of injection, suggests that the inhibition of the gonads by melatonin was incomplete, and that the melatonin in inhibiting an increase rather than directly causing a regression. If the latter were true, we would expect a decrease in the gonad size following melatonin injection.

The histological observations on the testis substantiated the data from the male GSI values. Despite the fact that absolute differences were not found, there was a marked tendency for reduced filling of the tubules by spermatozoa and spermatids in the testis of melatonin injected males. Nevertheless, all stages of spermatogenesis were present in all groups of males. The histological appearance of the initial controls was not observed, but the reduced size and reduced amount of material within the tubules of the melatonin injected males indicates an inhibition of spermatogenesis. The presence of all stages of spermatogenesis shows that the inhibition was not complete.

The histological appearance of the ovaries varied considerably within any one experimental group but no differences were
observed between any two groups. All stages of maturation were present within the ovaries of each group. Therefore, although melatonin inhibited the overall growth of the ovaries, it did not specifically inhibit any one step in ovogenesis or completely inhibit the process of maturation.

The effects of melatonin injection on the interrenal nuclear diameter, the relative height of the thyroidal epithelium, and the diameter of the pituitary gonadotrophs are summarized in Table IX. Melatonin had no effect on the relative height of the thyroidal epithelium. But the melatonin injected animals had significantly ($P < .025$) smaller interrenal nuclei and significantly ($P < .01$) larger gonadotrophs than the solvent injected group.

These findings, together with those presented earlier, suggest that the inhibitory action of melatonin on the gonads is exerted by way of the pituitary gland. The increased size of the gonadotrophs could indicate that these cells are producing but not secreting gonadotropic hormone. Conversely, melatonin may be inhibiting the action of gonadotropin on the gonads with the result that the gonadotrophs become hyperactive.

Decreased interrenal nuclear diameters following melatonin injection shows that the action of melatonin is not limited to the reproductive system but also affects the electrolyte system through the interrenal tissue. Nevertheless, melatonin did not affect the thyroidal tissue and is therefore not a general pituitary inhibitor.
TABLE IX

Effect of melatonin injection on interrenal nuclear diameter, relative thyroid epithelial cell height and gonadotroph diameter of goldfish. Tabulated values are means.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Solvent Injected</th>
<th>Melatonin Injected</th>
<th>Significance t-test (df)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interrenal nuclei (microns)</td>
<td>5.52</td>
<td>5.15</td>
<td>$P &lt; .025$ (4)</td>
</tr>
<tr>
<td>Thyroid epithelium</td>
<td>1.40</td>
<td>2.04</td>
<td>NS (4)</td>
</tr>
<tr>
<td>Gonadotroph diameter (ocular units)*</td>
<td>5.92</td>
<td>6.93</td>
<td>$P &lt; .01$ (49)</td>
</tr>
</tbody>
</table>

*1 ocular unit = .92 microns
DISCUSSION

The localization of melatonin within the pineal organ of fish corroborates the results of other workers. Quay (1965b) found that the enzyme hydroxyindole-O-methyl transferase, required for melatonin synthesis, is present in the pineal gland of fishes. Recently, Oguri et al. (1968) demonstrated that $^{14}$C-labelled 5-hydroxytryptophane is taken up by the pineal organ of trout in greater quantities than any other tissue studied. As this amine is a precursor of serotonin, and therefore a precursor of melatonin, they concluded that the photoreceptive pineal organ of the trout *Salmon irideus* (Dodt, 1963; Morita, 1966) also produces melatonin. Quay (1965) also found HIOMT in the retina of the fish but in the present study melatonin could not be localized in this tissue taken from salmon. Whether this means that the retina produces melatonin but does not store it, or whether the presence of the enzyme HIOMT does not always result in melatonin synthesis is not known. Nevertheless, the high levels of melatonin found in the pineal organ of the salmon indicate that in this species the pineal gland is a principal source of melatonin.

The level of melatonin found in mature salmon (0.68 µg/g) approximates the level found in rats (0.40 µg/g) by Prop and Ariëns Kappers (1961) but exceeds those reported in the pineal of cows (Learner et al., 1960), pigeons (Quay, 1966) and weavers (Ralph et al., 1967). In immature salmon, the pineal melatonin store is much greater (4.49 µg/g) than in the pineals of mature
salmon, and may be related to the inhibition of maturation. Be that as it may, the marked changes in pineal melatonin stores between mature and immature salmon might also be related to the aquatic environment. The higher melatonin level was found in the pineals of ocean dwelling salmon and the lower level was found in mature salmon which had moved into fresh water. Melatonin has previously been implicated in adrenal physiology (Fraschini et al., 1968), and results in a reduction of adrenal weight when implanted into the median eminence of male rats. Similar results were obtained in the goldfish (Table IX). Melatonin injected animals had smaller interrenal nuclei than solvent injected animals. How melatonin affects the internal nuclei is not clear, but the possibility that melatonin levels are related to the osmotic environment of fishes must be considered.

Notwithstanding the possibility that the lower pineal melatonin store in mature as opposed to immature salmon is related to the change from sea water to fresh water, the decrease corresponds with what one would expect if melatonin is acting as a gonadal inhibitor.

The data from goldfish injected with melatonin shows a direct relationship between melatonin and the reproductive system, and therefore corroborates the alterations in melatonin level between immature and mature salmon. The effect of melatonin was to inhibit the increase in gonad size of male and female goldfish when they were exposed to increased day length. The inhibition of the gonads was accompanied by an increase in the size of the pituitary gonadotrophs, and, in the male, a decrease in
the quantity of spermatozoa and spermatids within the tubules of the testis.

Previous reports from mammals concur with the findings reported here. Wurtman et al. (1963) have reported a delay in the vaginal opening and a reduction of ovarian weight following melatonin treatment in rats, and Wurtman and Axelrod (1965b) found a decreased incidence of estrus in melatonin treated adult rats. In pursuance of the same problem, Adams, Wan and Sohler (1965) inhibited the weight increase in the ovaries of rats between days 35 and 49 postnatal and found that these animals had smaller pituitaries with higher levels of luteinizing hormone (LH). This latter finding is supported here by the larger gonadotrophic cells found in the melatonin injected goldfish. Unfortunately, data prior to melatonin injection was not collected so that it is not known if the larger gonadotrophs in the melatonin injected animals resulted from an actual increase in size, or a failure to decrease following exposure to long photoperiod. Nevertheless, the principal effect of melatonin in mammals is suggested to be that of inhibition of LH secretion (Motta et al., 1967; Fraschini et al., 1968).

Among male mammals, melatonin selectively decreases the weight of the prostate and seminal vesicles which are largely under the control of LH but does not reduce the weight of the FSH dependant testis (Wurtman et al., 1963; Kappers, 1964; Moskowska, 1965). As an LH like hormone is thought to be the major, if not the only fish gonadotropin (Yamazaki and Donaldson, 1968), the effect of melatonin injections on the pituitary
gonadotrophs and the marked effect on the gonads is probably not fortuitous. It is tempting to suggest that the data obtained in this study supports the hypothesis that a single fish gonadotropic hormone serves the function carried out by two hormones in mammals, and that the fish gonadotropic hormone has characteristics common to both mammalian hormones.

The data reported here, as well as that available in the literature (Fraschini et al., 1968) indicate that melatonin acts by inhibiting the stimulation of the gonads rather than directly producing regression of them. Although melatonin prevented a significant increase in the size of the gonads during exposure of the goldfish to long photoperiod, the gonads did not decrease in size. From this fact, as well as the effect of the melatonin on the gonadotrophs, it is concluded that the action of the melatonin is centered in the pituitary gland or the hypothalamic centers governing pituitary function.

From studies with mammals, a direct action of melatonin on the pituitary appears unlikely. Moszkowska (1965) found that melatonin alone was unable to produce any effect on in vitro pituitary cultures but that it did reduce the activation of this tissue by hypothalamic extracts. Recently, Fraschini et al. (1968) report that melatonin, implanted directly into the pituitary tissue of rats, does not change the level of pituitary LH but that implantation of melatonin into the median eminence or reticular substance of the midbrain results in a reduction of pituitary LH stores and a decrease in the level of circulating LH. Hence, the effects of melatonin reported here are
probably the result of some action on the hypothalamic centers which regulate the synthesis or secretion of pituitary gonadotropin. The larger size of the gonadotrophs in the pituitary glands of melatonin-injected goldfish indicates an increase in gonadotropin synthesis or storage. The increase in synthesis appears unlikely however, as melatonin implants decrease the level of LH in the pituitary glands of mammals (Fraschini et al., 1968). Further, inhibition of release of gonadotropin is indicated by the fact that melatonin treated rats do not undergo a decrease in pituitary LH stores during puberty. Whatever the mechanism, it appears as though melatonin inhibits the stimulatory effect of long photoperiod on the gonads.

Failure to detect any effect of melatonin on the apparent thyroidal activity in goldfish might result from the technique employed. The histological approach used is thought by some to be an unreliable criterion of thyroidal activity (Matty, 1960; Eales, 1963). However, as the principal criticism of this technique is a lag between the thyroidal cell height and the secretory activity, and as the animals tested in this study were allowed 50 days for changes to occur, the results obtained were taken as indications of thyroidal activity. The results of the study on the effect of melatonin injection on the thyroidal tissue are at variance with those of Panda and Turner (1968) who report that in rats, melatonin has an action similar to a goitrogen and produces a decrease in thyroidal secretory rate, but agree with the findings of Thieblot et al., (1966). The implication is that in the goldfish, the action of melatonin is
specific for certain pituitary functions and does not generally inhibit hypophyseal functions.

In conclusion, the following picture emerges. Melatonin is present within the pineal of both mammals and fish. The level of melatonin is related to the state of reproductive maturity and exogenous melatonin can inhibit gonadal growth in both groups of animals. It is therefore postulated that with regards to the reproductive system, the pineals of mammals and fishes are functionally similar. Melatonin, present in high concentrations in the pineal of immature salmon, may prevent the maturation of the gonads by acting on certain hypothalamic centers which regulates gonadotropin release. At some time during the reproductive cycle, the melatonin level in the pineal decreases resulting in an increase in gonadotropin secretion which in turn stimulate an increase in gonad size and reproductive maturation. The melatonin, or rather the decrease in pineal melatonin stored, then acts as a trigger by releasing greater quantities or allowing enhanced production of gonadotropic hormone.
SECTION III

EFFECT OF PINEALECTOMY ON THE REPRODUCTIVE AND ENDOCRINE SYSTEMS IN GOLDFISH

INTRODUCTION

A secretory role of the fish pineal organ has been suggested. Holmgren (1958), Rasquin (1958), and Altner (1965) described a secretory role based on apocrine secretion (Hafeez and Ford, 1967). Cytological studies have indicated a high cellular metabolism (Palayer, 1958; Holmgren, 1959a) and a well-developed golgi complex (Rüdeberg, 1968b) in fish pineal cells. Though most authors agree that this secretion enters the third ventricle (Rasquin, 1958; Holmgren, 1959b; Hafeez and Ford, 1967), Friedrick-Freksa (1932) and Holmgren (1959) suggest that some of the pineal secretion passes directly into the blood capillaries. But relatively few investigations on the possible function of pineal secretion have been reported, and the results of these studies are contradictory and inconclusive.

Pflugfelder (1953, 1954, 1956a) suggested that within the teleosts a pineal-pituitary relationship exists. His observations, based on pinealectomized guppies Lebiasites, indicated hypertrophy of the adenohypophyses and thyroid, decreased growth rate, a slight acceleration in the appearance of secondary sex characteristics in young males, an increase in the activity of the interrenal cells, and a disturbed calcium metabolism resulting in spinal curvature. Subsequently, the same author
(Pflugfelder, 1956a) found that the thyroidal hypertrophy was diminished by epiphysan or thyroxin and that pinealectomized goldfish had alterations in the pars distalis and thyroid tissue (Pflugfelder, 1964).

Holmgren (1959b) reported skeletal abnormalities in pinealectomized Fundulus heteroclitus which were attenuated by administration of beef pineal extracts. In the same study, he found that pinealectomy affected a decreased radiocalcium uptake while an increase was apparent following pineal extract treatment. Weisbart and Fenwick (1966) found no differences in blood plasma levels of calcium following pinealectomy of goldfish.

Contrary to the report by Pflugfelder (1964), Pang (1968) found a decreased thyroidal cell height in pinealectomized Fundulus and Rasquin (1958), Holmgren (1959b) and Peter (1968) found that pinealectomy had no effect on the thyroidal tissue of various fish. Peter (1968) also reports no effect of pinealectomy on the interrenal cells of pinealectomized goldfish. Schoherr (1955), Rasquin (1958) and Peter (1968) also found that pinealectomy had no effect on the gonads of fish. Pang (1967), however, described a delayed appearance of nuptial coloration in pinealectomized Fundulus but reported that controls had a smaller gonad size. Whether this means that the controls underwent gonadal atrophy, or that his pinealectomized fish showed gonadal hypertrophy is not clear. Nevertheless, the possible internal secretion of the pineal cells and the diverse effects following pinealectomy in some fish suggests that the
pineal organ of the fish is an endocrine organ.

Previous studies on the effect of pinealectomy on the gonads of fish have not taken into consideration the marked reproductive cycles of the experimental animals. Pang (1967) found differences in the degree that pinealectomy affected growth rate and gonad size when pinealectomy was performed during the initial phase of growth and sexual maturation in *Fundulus* as opposed to when the same animals were pinealectomized during the height of growth and reproduction. This indicates that the functions of the pineal organ in fish varies between different times of the reproductive cycle, among animals of different ages, or both. Therefore, in testing the effect of pinealectomy on the reproductive system of fish, the annual yearly cycle must be considered and the animals should be tested at various stages throughout the cycle.

The principal aim of this study was to examine the effect of pinealectomy on the gonads in goldfish. To achieve this, the normal reproductive cycle of the goldfish was estimated under laboratory conditions. Studies were then undertaken to find when the reproductive cycle was most affected by increasing day length. Finally, the effect of pinealectomy was tested at different times of the reproductive cycle and under different photoperiods.

Subsidiary studies were performed to test the effect of pinealectomy on the thyroidal tissue, interrenal tissue, blood plasma osmotic concentration and blood plasma levels of Ca\(^{++}\), Cl\(^{-}\), and Na\(^{+}\).
MATERIALS AND METHODS

All goldfish used, together with their source and maintenance prior to the start of experiments have been described in Section I, but in the present experiment the fish were allowed to swim freely within the holding tank. The operative procedure of pinealectomy is to be found in the same section. The histological procedures and quantification of histological results are described in Section II. Unless specifically mentioned, the fish were maintained in water held at 20°C during the experiments and were subjected to controlled light regimens.

Estimation of Reproductive Cycle

To estimate the seasonal variation in gonad size of goldfish held under laboratory conditions, a minimum of 10 male and 10 female goldfish were sampled during each of six pre-selected months. The photoperiod to which these fish were exposed varied throughout the year and at any given time approximated that of the natural daily photoperiod.

Effect of Photoperiod on the Gonad Size at Different Times of the Year

To establish the effect of photoperiod at various times of the year, groups of fish were exposed to photoperiods of 1L (one hour of light alternating with 23 hours of darkness), 8L (eight hours of light alternating with 16 hours of darkness),
16L (16 hours of light alternating with 8 hours of darkness), or 24L (continuous light) during the intervals Dec. 23 - March 21, April 5 - May 20, Aug. 1 to 10, and Oct. 28 - Dec. 14. The fish were killed and the mean gonad size (GSI = gonad weight/body weight x 100) for male and female goldfish were calculated separately. The mean GSI values calculated were based on an average sample size of 11 (Range: 4-18).

Effect of Pinealectomy on the GSI

Table X lists the duration and timing of experiments testing the effect of pinealectomy on the GSI of male and female goldfish at different times of the year and under different photoperiods and lists other parameters when tested. Since many of the data revealed no significant differences between the treatments they will not be included in the results. However, the size and scope of each experiment together with individual sample sizes are given (Table X). All experiments were carried out at the photoperiods indicated in Table X. Experiments 1, 2, and 3 were performed in standing water at 20 C and Experiment 4 was performed in water at 13 C ± 1.0 C into which fresh water was allowed to flow (overflow was removed by a standpipe at one end). Each experiment consisted of groups of pinealectomized, sham-pinealectomized and unoperated controls. The control and experimental fish for any one experiment and photoperiod were maintained together in aquaria of approximately 350 liters filled to capacity with dechlorinated water. Equal parts of NaCl and CaCl₂ were added in sufficient
TABLE X  Scope and timing of pinealectomy experiments.

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<th>Experiment number and Duration</th>
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1. Hours of light the animals were exposed to each day.
2. Treatment; P, pinealectomized, S, sham-pinealectomized, N, unoperated controls.
3. Interrenal nuclear diameters were measured.
4. Relative height of thyroidal epithelium was determined.
5. Temperature of water was 13°C as opposed to 20°C for all other experiments.
quantity to bring the final salt concentration to 0.2%. In the case of Experiment 4, the salts were added daily to replace loss through the overflow pipe. Treatment with salt, together with the addition of Malachite Green (one drop of 1% aqueous solution to each 100 liters of water), adjusted for the low level of dissolved solids in Vancouver city water, and prevented *Saprolegnia* infection of the goldfish.

In addition to the investigations listed in Table X, 10 pinealectomized, 10 sham-pinealectomized and 10 unoperated control fish were placed in 50% sea water and equivalent numbers of the same groups were placed in fresh water. After two weeks the plasma osmolar concentration of Ca\(^{++}\), Cl\(^{-}\) and Na\(^{+}\) were determined together with the melting points of the plasma from the different groups.

A. Collection of Plasma

Goldfish were anaesthetized as described earlier, dried lightly with a paper towel, and descaled from the region posterior to the vent. The caudal peduncle was severed posterior to the anal fin and the blood was allowed to run down a heparinized capillary tube. The tubes were sealed with a mixture of paraffin wax and petroleum jelly (1:1) and centrifuged at 600X g for two minutes. The capillary tubes containing the blood were cut at the junction between the plasma and the cells and small samples of plasma were taken.
B. Analysis of Plasma

Melting points of plasma were determined with a melting point determination apparatus designed by Ramsay and Brown (1955). Small samples of the plasma were drawn into fine capillary tubes (#40090, Central Scientific Co., Vancouver). These samples were enclosed by paraffin oil to prevent evaporation and were kept over solid CO$_2$ until analysis. Melting points of blood were determined on the day of collection.

Concentrations of the cation Na$^+$ were analysed by emission flame photometry using a Unicam SP900A Spectrophotometer (Unicam Instruments Ltd., Cambridge, England). The plasma sample was diluted 5000X in polyethylene vials (10 ml) using Drummond Microcaps (Drummond Scientific Co., Broomall, Pa.). The vials were stored over solid CO$_2$ prior to sample analysis. Care was taken to analyse only after the sample had reached room temperature. As a check on the quantitative accuracy of this cation determination, dilutions of Harleco Serum Control (#64098 Harlman-Leddon Co., Philadelphia, Pa) were also analysed by flame photometry.

Chloride concentrations were measured potentiometrically on 1 microliter (0.001 ml) plasma samples diluted in 20 microliters of 50% acetic acid after the method of Ramsay (1955). A Radiometer pH Meter 25SE (Radiometer, Copenhagen, Denmark) and a Misco Vibrating Stirrer (#1385 Microchemical Specialities Co., Berkeley, California) with a 60 ohm resistor between the transformer and the solenoid was used.

Calcium concentrations in plasma were measured fluoro-
metrically on 20 microliter samples of plasma. The determinations were made on a Turner Model III Fluorometer (G.K. Turner Associates, Palo Alto, California) using the method described in the Turner manual with the exception that Harleco Serum Control was used to obtain standard curves.

**Statistical Methods**

With the exception of the histological appearance of the gonads, on which no quantifiable observations were made, all groups of data obtained on any parameter from any one experiment were tested separately by analysis of variance. When significant differences (P < .05) were found, individual differences between means were tested with Tukey's w procedure.
RESULTS

Seasonal Variation in Gonad Size

Goldfish exhibited marked seasonal differences in gonad size (Figure 4). The maximum GSI value for both males and females was found during the month of May, and coincided with the normal spawning period of these animals (Yamazaki, 1965). In nature, this peak would decline rapidly during ovulation or spermiation (Yamazaki, 1965). Under laboratory conditions the rapid decline failed to appear; the gonads regressed slowly during the summer, stabilized at a small size during the winter, and underwent renewed growth in March. Thus, although seasonal variations in the GSI of goldfish held under artificial conditions only partially reflect the normal annual cycle, the general trend approximates that seen in nature. Further, the experimental results allow inferences to be drawn about the general state of the animal's reproductive physiology at the time of the various pinealectomy experiments.

Seasonal Variation of the Effect of Photoperiod on the Gonad Size

Figure 5 summarizes the effect of different photoperiods on the GSI's of male and female goldfish during different times of their annual reproductive cycle. With few exceptions, the yearly cycle described previously persists regardless of the photoperiod under which the animals were held. Further, only during the period April 5 to May 20 (Fig. 5) was a marked and
Figure 4. Seasonal variation of the GSI in male and female goldfish under laboratory conditions. Values are means ± 95% confidence interval, each mean is based on 10 animals.
The graph shows the GSI (x±95% confidence interval) for females and males over the course of the year. The graph indicates a peak in May for both males and females, with a notable increase in GSI for females compared to males during this period. The GSI for both groups decreases significantly after May, with females maintaining a higher GSI through the summer months compared to males.
Figure 5. Seasonal variation of photoperiod effect on the gonad size of male and female goldfish representing gonads as percent of body weight (GSI). (1L, 1 hour of light alternating with 23 hours of darkness; 8L, 8 hours of light alternating with 16 hours of darkness; 16L sixteen hours of light alternating with 8 hours of darkness; 24L, continuous light). Animals were held under the specified photoperiod for a minimum of 48 days.
consistent increase in the gonad size evident with increasing day length. Females also showed a consistent increase in ovary size with increased photoperiod during the period from Dec. 23 to March 21 while the males did not. This increase was not of the same magnitude as that seen later in the spring. Thus, increased light exposure acts as a gonadal stimulus during only part of the year.

In summary, the size of the gonads in male and female goldfish is under at least a dual control. The first is an endogenous rhythm which is not dependant upon environmental cues such as temperature and photoperiod and functions continuously throughout the year. The second shows a cyclical responsiveness to environmental photoperiods, the peak responsiveness coinciding with the peak gonadal growth under the endogenous cycle.

Effect of Pinealectomy on the Gonad Size

Throughout the periods June 25 to Aug. 8, Oct. 28 to Dec. 14, and April 5 to May 20, pinealectomy had no effect on the gonad size in male or female goldfish, regardless of the photoperiod under which they were held. During the longer interval of Jan. 9 to May 3, pinealectomy enhanced the size of the gonads in both male and female goldfish. The data from this latter experiment are summarized in Tables XI and XII. Table XI lists the mean GSI values for groups of 18 male or female pinealectomized, sham-pinealectomized and unoperated control goldfish, and Table XII presents a two way analysis of variance.


**TABLE XI**

Effect of pinealectomy on the gonad size of male and female goldfish during the period Jan. 9 to May 3.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pinealectomized</td>
<td>2.56</td>
<td>4.28</td>
</tr>
<tr>
<td>Sham-pinealectomized</td>
<td>1.08</td>
<td>2.18</td>
</tr>
<tr>
<td>Unoperated controls</td>
<td>0.91</td>
<td>1.50</td>
</tr>
</tbody>
</table>

*Each mean is taken from 18 fish held at 13°C ± 1°C and under an eight hour photoperiod. Tukey's $w_{.01} = 1.32$. 
TABLE XII

Analysis of variance of the GSI values of 18 pinealectomized, sham-pinealectomized, and unoperated control goldfish of both sexes during the period Jan. 9 to May 3.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>1</td>
<td>35.20</td>
<td>35.20</td>
<td>27.93</td>
</tr>
<tr>
<td>Treatment</td>
<td>2</td>
<td>99.40</td>
<td>49.70</td>
<td>39.44</td>
</tr>
<tr>
<td>Interaction</td>
<td>2</td>
<td>5.74</td>
<td>2.87</td>
<td>2.28</td>
</tr>
<tr>
<td>Error</td>
<td>102</td>
<td>128.72</td>
<td>1.26</td>
<td></td>
</tr>
</tbody>
</table>

Therefore Sex and Treatment are highly significant.
Interaction is not significant.
on the data from which the means were taken. A significant difference at the 99% level was calculated at 1.32 (Tukey's w procedure). Using this value to test the differences between different groups of males and females it was found that the pinealectomized males and females had significantly ($P < .01$) larger GSI values than the corresponding sham-operated or unoperated controls. The sham operation did not produce any significant effect.

**Effect of Pinealectomy on the Histology of Reproductive and Endocrine Tissue**

Pinealectomy did not effect any changes in the diameter of the interrenal nuclei, cell height of thyroidal epithelium, or the general histological appearance of the testis or ovaries in any of the experiments in which these tissues were studied (Table X).

**Effect of Pinealectomy on the Electrolyte System**

The melting point of blood plasma, the levels of the cations $Na^+$, and $Ca^{++}$, and the level of the anion $Cl^-$ were not altered by pinealectomy under any of the conditions tested. Occasionally variations in these parameters resulted from differences in the environment (photoperiod, salinity) but the variations were not affected by pinealectomy.
DISCUSSION

Pinealectomy of mammals (Wurtman, Altschule and Holmgren, 1959; Motta, Fraschini and Martini, 1967), birds (Izawa, 1923; Shellabarger and Breneman, 1950), and lizards (Stebbins, 1960) produces gonadal hypertrophy. Under specific conditions, this result was duplicated in the present study on goldfish.

Reported effects of pinealectomy on the reproductive system of fish are contradictory. Pflugfelder (1956a) demonstrated a relationship between the pineal organ and reproductive maturation in male Lebistes reticulatus, but his findings have not been substantiated in Gasterosteus aculeatus (Schonnherr, 1955), Astyanax mexicanus (Rasquin, 1958) or previously in the goldfish Carassius auratus (Peter, 1968). This discrepancy may be accounted for by species differences in the function of the pineal organ or by differences in experimental design.

In the present study the goldfish were found to have an endogenous cycle in gonad size which persisted under all photoperiods tested. Further, a marked increase in gonad size following prolonged daily light exposure was evident only during the interval within which the gonads would endogenously increase in size. Based on these findings, it was postulated that the effect of pinealectomy on the reproductive system of the goldfish would vary during the course of the endogenous cycle or alter the endogenous cycle.

The GSI values obtained from pinealectomized, sham-pinealectomized and control animals held under different photoperiods
at various times of the reproductive cycle indicate that the pineal gland does not control the endogenous cycle; that is, removal of the pineal gland did not alter the timing of the whole endogenous cycle. Pinealectomized goldfish had maximum and minimum GSI values at the same time as the sham operated and control fish and the operation had no effect during most of the year. Pinealectomy did, however, enhance the size of the gonads when the operation was performed just prior (Jan. 9) to the time when the gonads endogenously increased. Similar results were not obtained from animals pinealectomized during the time (April 5) when the gonads were already increasing endogenously. The time at which pinealectomy was effective also coincided with the period during which the increasing photoperiod is capable of stimulating gonadal enlargement. These results indicate that the pineal gland is associated with the timing of the responsiveness of the hypothalamous, pituitary or gonads to increasing day length, so that removal of the pineal gland lowers the threshold of day length required for gonadal stimulation or enhances the photoperiodic efficiency. Thus, the data indicate a functional relationship between the pineal organ, photoperiod, and reproductive development. This relationship has been demonstrated in mammals (Hoffman and Reiter, 1966; Roth, 1964; Reiter and Hester, 1966).

The lower GSI values of all control groups held at 13°C as opposed to those held at 20°C under eight hours of light per day and at the same time of year suggests a temperature effect. It is well documented (Pickford and Atz, 1957) that temperature
has a marked effect on the reproductive system in fish. Butler (cited in Pickford and Atz, 1957) found that goldfish held at \(9 \text{ C}\) possessed testes in a resting state while those at \(21 \text{ C}\) had all stages of spermatogenesis present within their testes. Thus the results obtained by pinealectomy of fish at \(13 \text{ C}\) may indicate a change in the thermal requirements for gonadal growth. However, as pinealectomized male and female goldfish had larger GSI values (2.56 and 4.28 respectively) at \(13 \text{ C}\) than normal animals at \(20 \text{ C}\) (1.90 and 3.80 for males and females) at the same time of year and under the same photoperiod, the increase in the GSI following pinealectomy is more than just a change in thermal requirements.

Data concerning the effects of pinealectomy on the electrolyte system and the interrenal tissue shows no relationship between the pineal gland and this system and fails to corroborate the disturbed calcium metabolism seen following pinealectomy (Pflugfelder, 1963; Holmgren, 1959b). These authors based their opinions on skeletal abnormalities or changes in uptake of radioactive calcium, both of which could occur without alterations in the plasma \(\text{Ca}^{++}\) levels.

Failure to find any alteration in the interrenal nuclei conforms with the findings of Pflugfelder (1964) and Peter (1968) on the goldfish, and Pang (1967) on the killifish \textit{Fundulus heteroclitus}, but is at variance with the reported increased activity of interrenal cells in pinealectomized guppies (Pflugfelder, 1953). However, the results agreed with those obtained from melting points and ionic levels of the plasma since they
also failed to respond to pinealectomy.

The hyperthyroidism reported by Pflugfelder (1964) on the goldfish and guppy (Pflugfelder, 1953, 1954, 1956a) following pinealectomy was not substantiated by this study. This result agrees with those of Rasquin (1958) on Astyanax mexicanus, Pang (1967) on Fundulus heteroclitus and Peter (1968) on goldfish.

All data considered, the pineal gland of the goldfish appears to exert an inhibitory influence specifically on the reproductive system. The endogenous rhythm establishes the phase of sensitivity to photoperiodic illumination. The photoperiod sets the level of photic stimulation. But it is the pineal gland which sets the threshold level of illumination during the sensitive phase of the endogenous rhythm.

During much of the year, the phase of the endogenous cycle is such that the reproductive system is insensitive to increasing daily light exposure. At this time, the inhibitory action of the pineal organ is superfluous. However, as the endogenous cycle enters the photosensitive phase the pineal's inhibitory influence becomes operational. This inhibitory action may slow down the development of the gonads resulting from the increasing photoperiods which begin in mid-winter and maintain the gonads at a low level of development until the photoperiod has reached its effective threshold.

Such a system would account for the accelerated increase in the size of the gonads during the month of April as opposed to the slow increase in the preceding three months during which the photoperiod is increasing. The pineal does not alter the
phase of the endogenous cycle, but it does inhibit the response to the increasing photoperiod.

This synchronization of the gonadal development with the photoperiod could have a marked effect on the efficiency of the reproduction of the population as a whole. If the endogenous cycle and the photoperiod operated alone, individual variations in the timing of the phases of sensitivity would produce individual responses to photoperiod and animals would mature at different times. By adding the pineal gland to the system, two synchronizing mechanisms are at work: (1) the endogenous cycle must be in the phase which is sensitive to increasing photoperiods, and (2) the photoperiod must increase to a certain threshold to deactivate the pineal's inhibitory influence.
The pineal organ of fishes has been described as a photosensitive structure (Young, 1935; Breder and Rasquin, 1950; Dodt, 1963) intimately associated with phototactic behaviour (Breder and Rasquin, 1947; Hoar, 1955; Pang, 1966). It has also been described as a secretory organ (Holmgren, 1918a,b; Holmgren, 1958b; and Altner, 1965) of undetermined function. The secretion is generally regarded as entering the cerebrospinal fluid (Friedrick-Freksa, 1932; Hafeez and Ford, 1967) although Friedrick-Freksa (1932) and Holmgren (1959a) suggested that some of the pineal secretion is taken up by pineal blood vessels. Now it appears that both functions, photoreception and secretion, occur within the fish pineal organ (Palayer, 1958; Holmgren, 1959a; and Rüdeberg, 1968b) and that one or both of these are related to the endocrine system (Pflugfelder, 1953, 1954, 1956a, 1964; Pang, 1967) though such a relationship is denied by some (Rasquin, 1958; Holmgren, 1959b; Peter, 1968; Peter and Gorbman, 1968).

Within two closely related species of Pacific salmon (Oncorhynchus nerka and O. tshawytscha) the pineal organ was found to function as a photoreceptor (Hoar, 1955) and, in this study, to produce the hormone melatonin. Further, in various studies on the rainbow trout Salmo gairdnerii irideus, Dodt (1963) and Morita (1966) found action potentials in the pineal body which could be altered by light. Quay (1965b) localized within the pineal organ the enzyme responsible for melatonin synthesis, and
Oguri et al. (1968) reported that radioactive 5-hydroxytryptophane, a precursor of melatonin, was taken up in greater amounts into the pineal tissue than any other tissue studied.

The present study indicates that the pineal body of the goldfish is involved in neural phenomena as exemplified by the effect of pinealectomy on various behavioural responses to light and on swimming activity. Further, the pineal gland appears to have endocrine effects since its removal during part of the year results in an increase in the size of the gonads. This latter effect appears to be related to the photosensitive nature of the pineal organ or to responses to photic illumination. Pinealectomy only produces a response of the gonads during the time when photoperiod is capable of stimulating the gonads. Further, melatonin, the presumed pineal hormone, is capable of inhibiting this light induced increase in gonad size. This leads to the conclusion that the pineal organ of the fishes has two functions which are under a single control; that is, the pineal has neural as well as endocrine functions, both of which are related to the photoreceptive nature of the pineal organ.

The behavioural effects and the endocrine effects of the pineal are both associated with its photoreceptive ability. Within the mammals, the pineal has lost its direct photosensory role (Kappers, 1965) and is devoid of the photoreceptive structures (Kelly, 1962) present within the fish pineal organs. Nevertheless, the pineal organ of mammals remains in part under the control of environmental illumination (Fiske et al., 1962; Quay, 1963b,c; Snyder, Zweig and Axelrod, 1964; Zweig et al.,
by way of the eyes (Wurtman, 1967; Reiter and Hester, 1966) and sympathetic nervous system (Snyder et al., 1966; Reiter and Hester, 1966) and as such is functionally similar to that of the fish pineal organ. The differences between the direct effects of light on the pineal gland of fishes and the indirect effects of light on the pineal gland of mammals represent only a change in the pathway by which light affects the glands rather than a difference in the function of the glands themselves.

The data from the present investigation indicate that the effects of the pineal on the reproductive system are mediated by melatonin acting on the pituitary gland. From previous studies on mammals (Fraschini et al., 1968) it is suggested that the effects of melatonin on the pituitary are due to the presence of receptors within the hypothalamus which are responsive to melatonin. Melatonin administration in mammals, aside from the endocrine effects (Wurtman et al., 1961) also produces neural effects (Fiske and Huppert, 1968) including the elevation of the serotonin concentration in the mid brain but not in the cerebral cortex, olfactory bulbs or tubercle (Anton-Tay et al., 1968). As noted earlier (Fig. 2) removal of the pineal organ of goldfish resulted in increased swimming activity but did not alter whole brain serotonin level (Table V). But the possibility of localized changes in serotonin level was mentioned. If the changes in serotonin level in the mid brain result from melatonin, then removal of the pineal, which may be the principal source of melatonin, could result in a decreased serotonin level in the mid brain of goldfish, which could then alter local
neural activity and result in the increased swimming activity. On the other hand, the increased swimming activity could have resulted from an increased production of gonadal steroids following pinealectomy. This seems unlikely, however, as it would not account for the even greater increase in swimming activity seen in blinded goldfish. Further, the increase in swimming activity following pinealectomy was observed in goldfish which were pinealectomized during that part of the year when pinealectomy was found to have no effect on the size of the gonads.

Thus, the functions of the pineal gland, both neural and endocrine may depend upon its production of melatonin. Further, the neural effects may be directly related to the endocrine effects. Melatonin, by altering the level of serotonin, could effect changes in the neuroendocrine activity in the midbrain (Anton-Tay et al., 1968) which might then alter the activity of the hypothalamo-hypophyseal complex resulting in altered pituitary function.

In reexamining the working hypothesis of pineal functions of fishes in the general introduction it is found that, with certain limitations, no reasons were found to reject them and the evidence was strongly in favour of accepting them. The pineal gland of goldfish does mediate behavioural responses to environmental illumination. It contains melatonin in the salmon and melatonin has the hormonal like quality of inhibiting gonadal growth in the goldfish. Further, removal of the pineal organ in the goldfish, which presumably contains melatonin, enhances the size of the gonads when the operation is performed.
prior to the onset of the endogenous gonadal growth initiated in the early part of the year. But pinealectomy has no effect on the gonads at other times of the year. Therefore, the pineal gland is associated with the reproductive system and serves as one of the channels by which the reproductive system is influenced by the environmental illumination.
SYNOPSIS

Now it appears that the pineal body of fishes is not a functionless, evolutionary vestige of a primitive third eye. On the contrary, the evidence suggests that it performs a dual function and that both functions depend upon light reception and possibly melatonin synthesis. Melatonin, produced within the fish pineal organ governs local neural activity within the brain which in turn controls neuroendocrine activity, behavioural responses, or both. In the mammals, the pineal has lost its direct photosensitivity but remains in part under the control of environmental illumination and is in this way similar to the pineal organ of fishes. Further, both the mammalian pineal organ and the fish pineal organ produce melatonin and this substance has antigonadal effects in both groups. Removal of the pineal organ in mammals or goldfish results in gonadal hypertrophy although in the fish this effect is limited to certain times of the reproductive cycle. The level of melatonin in the pineal of fishes and mammals is related to the reproductive system and is directly influenced by the endogenous cycle, conditions of light, or both. Thus, the pineal organ of fish is both a sensory and secretory structure. Its secretion affects both neural and endocrine activity so that functionally, the pineal glands of mammals and fishes are similar.
SUMMARIES

SECTION I

1. Pinealectomy, blinding, or both had the same effect in that all three treatments resulted in the loss of the photonegative response characteristic of wholly intact animals.

2. Goldfish with intact eyes but without a pineal displayed more swimming activity than intact animals. Those fish without eyes were more active than fish with eyes.

3. Although a causal relationship was not established, the amount of swimming activity in goldfish was lowest in those groups which possessed the highest level of whole brain serotonin. Pinealectomy did not affect the level of whole brain serotonin.

4. Pinealectomized goldfish with intact eyes were more responsive to light as a conditioned stimulus than were wholly intact controls. Blind goldfish, with or without a pineal, were not effectively conditioned to light although they were conditioned to sound.

5. Removal of the pineal organ resulted in a decrease in the voltage required to just produce a response but did not affect the response to the constant voltage used in the conditioning experiments.

6. Pinealectomy did not affect the conditioning of goldfish
SECTION II
1. Melatonin was localized in the pineal organ of the salmon and was found in higher concentrations in the pineal of immature salmon than in the pineal of mature salmon.

2. Melatonin injection in goldfish inhibited the endogenous increase in gonad size which occurs during the spring and inhibited the stimulatory effect of increasing daylength on the gonads.

3. Goldfish injected with melatonin had larger pituitary gonadotrophs and smaller interrenal nuclei than un.injected or solvent injected controls.

4. Melatonin injection effected no changes in thyroidal tissue.

SECTION III
1. Goldfish, held under natural photoperiods and at a constant temperature, exhibited a marked cycle in gonad size with both males and females having the largest gonads during May.

2. Increasing the photoperiod under which the goldfish were held stimulated the gonads only during the interval when the gonads would normally increase. Therefore, it did not effect the timing of the cycle as much as it augmented the normal increase.
3. Pinealectomy increased the gonad size only when performed near the time when the gonads would normally start to increase.

4. Other results of this report suggest that the pineal of the goldfish is not associated with the thyroid, interrenal tissue, plasma osmotic concentration, or the plasma levels of Ca^{++}, Na^{+}, and Cl^{-}.


Rudeberg, C. 1968b. Structure of the pineal organ of the sardine, Sardina pilchardus sardina (Risso) and some further remarks on the pineal organ of Mugil spp. Z. Zellforsch. 84:219-237.


