RHYTHMIC DIURNAL VARIATIONS IN THE OESTROUS PHENOMENA OF THE RAT AND THEIR SUSCEPTIBILITY TO LIGHT AND DARK

BY

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WITH 4 TABLES AND 4 FIGURES AND 1 TABLE IN THE TEXT

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CONTENTS.

1. Introduction.[1]

a. Diurnal rhythm of biological processes. A number of biological processes, notably metabolic, are subject to rhythmic variations synchronous with the natural astronomical alternation of day and night.

A classical example is the body temperature, which in man is lowest about 4—5 o'clock and highest about 18—19 o'clock.

In monkeys the curve of body temperature has been reversed by GALBRAITH and SIMPSON (1903; cited from LINDHARD, 1910) by reversing the daily light and dark periods.

Whereas human social life in civilized countries prevents complete reversal of night and day, in regard to factors other than light, so that even night-watches retain the normal curve of body temperature, LINDHARD (1910) in the polar night of North-East Greenland succeeded in reversing the curve of body temperature in 28 members of the Denmark Expedition by delaying bed-time once 4 hours and then 8 hours. He concludes that the curve of tem-

[1] Abstracts of the main conclusions have been read before the XV International Physiological Congress in Leningrad, 1935 (Summaries of Communications, p. 150), Dansk Naturhistorisk Forening (Danish Natural History Association, Copenhagen), 1935, and Biologisk Selskab (Biological Society, Copenhagen), 1935.

perature variations is determined by work and mode of living.

Other examples may be quoted in which, at least at first glance, the explanation appears less simple (cp. also Jores, 1935, and Müller, 1936).

Thus Hatlehol (1924) studied the blood-sugar curve of fasting diabetics and found that in heavy diabetes as a rule the blood-sugar fell in the course of the day, but rose during the night ("paradoxical rise").

The variation in regard to meals, muscular activity, and sleep, did not explain these rhythmic variations.

Pfaff and Balch (1897), et al., have found in patients carrying a biliary fistula, that the secretion of bile is subject to rhythmic diurnal variations. The same is true of the excretion of urea and urobilin. Also the diuresis is subject to such variations.

Attention has been directed to these phenomena particularly through the works of Forsgren (for references see his book from 1935). He has been engaged in studying the function of the liver, and from his publications it appears to be beyond doubt that, at least in rabbits and mice (cp. Ågren, Wilander, and Jorpes, 1931) the function of the liver is rhythmic, deposition of glycogen alternating with bile secretion; or, as Forsgren puts it, a phase of assimilation alternating with a phase of dissimilation in a 24 hours rhythm, and completely independently of, say, food consumption.

On the basis of Forsgren's studies, and in amplification of Hatlehol's work, Möllerström (1930) has found in non-fasting diabetics rhythmic diurnal variations in the blood-sugar and the excretion of sugar in the urine, apparently independent of exogenous factors.

Quite apart from their scientific importance, these rhythmic variations have a considerable practical bearing, e. g. in interpreting various functional tests and in the treatment of diabetics with insulin.

The underlying cause of these various rhythmic variations is unknown so far.

As regards the domain of sexual phenomena, a number of authors, on the basis of extensive statistical investigations, agree that deliveries in women are most frequent by night (JORES, 1935, p. 621—622); but it has received but little attention that also in the sexual phenomena of certain animals there are rhythmic diurnal variations.

Through our observation that the oestrous phenomena in the rat is subject to such rhythmic diurnal variations, an experimental basis is available for the study of their affectibility by various external factors.

Before passing on to a closer examination of this, it will be necessary to mention some details regarding the oestrous phenomena, which are necessary for the proper understanding of the following.

b. *The periodicity of oestrous phenomena.* In female mammals the sexual desire is generally restricted to definite periods of the mating season, the so-called periods of oestrus.

If no conception takes place during oestrus, the latter may recur a certain number of times (polyoestrous species), or there is no recurrence until the next mating season (monoestrous species).

In the wild state animals appear to have as a rule one mating season a year; but as well the number of mating seasons as the number of recurrences of oestrus (oestrous cycles) during a single mating season, may increase under

the influence of domestication. Thus, species which seem to be monoestrous in the wild state may become poly-oestrous when domesticated.

The interval between the oestrous periods within a mating season is called the dioestrous interval. The non-breeding period of the year when the generative organs are at rest, is called the anoestrous period, which in many mammals occupies the greater part of the year.

The number of recurrences of oestrus within a mating season and the length of the oestrous cycles in polyoestrous mammals, varies from species to species. In cattle and in the mare the length is 3 weeks; in primates, 1 month; in the guinea pig, 16 days.

Detailed descriptions of oestrus and its associated pheno-mena are to be found in the works of Heape (1901), Mar-shall (1922), and Parkes (1929).

The laboratory rat, with which the experiments reported on in this paper, are primarily concerned (albino Wistar stock), is, like the laboratory mouse, polyoestrous with an oestrous cycle of 4—5 days (Long and Evans, 1922).

These laboratory forms breed practically all the year round, although there seems to be most pregnancies in the spring and summer time.

Wang (1923, 1924) and Slonaker (1924) have shown that the spontaneous muscular activity of female rats with free access to revolving wheels, exhibits marked rhythmic changes in close relation to the oestrous cycle, the day of maximum activity coinciding with the day of oestrus.

The time relations of heat and anatomical oestrous changes in the female rat were characterized by Long and Evans (1922) as follows. The oestrus is preceded by the stage of prooestrus during which the follicles of the ovary

are growing large, the uterus becomes distended with fluid and the smear of the vaginal content shows epithelial nucleated cells only. Toward the end of this stage the rat may be in heat. In the stage of oestrus the follicles have attained their largest size, the eggs may maturate, uterus reaches its greatest distention, and then regresses; the vaginal smear consists of cornified cells only and the animal is in heat. In the next stage, metoestrus I, ovulation takes place, the vaginal smear consists of an abundant mass of cornified cells, and the animal has ceased to show signs of heat. Then follows the stage of metoestrus II, characterized by young corpora lutea, eggs in the oviduct, small follicles and invasion of leucocytes into the vaginal lumen. In the dioestrous interval corpora lutea continue to grow, while the other portions of the genital tract are in a stage of relative quiescence, and the vaginal smear consists of leucocytes, epithelial cells, and mucus.

The periodicity of the oestrous phenomena in the female rat can thus be conveniently studied in the intact animals by three methods: first, by recording the spontaneous muscular activity by means of revolving wheels to which the animals have free access, second by means of mating tests, which of course in the end is the most important, and third by vaginal smears.

c. *The relation of oestrus to day and night, according to previous authors.* The spontaneous muscular activity of the rat is confined to the nightly hours (RICHTER, 1922), both on the oestrous and the non-oestrous days of the 4—5 days oestrous cycle (SLONAKER, 1925). According to ISHII (1922, p. 313) and HEMMINGSEN (1933, p. 128—129), in the female rat on the day of oestrus heat is strongest toward midnight; and there seemed also (HEMMINGSEN,

1933, p. 129) to be a correlation between the cyclic vaginal changes and the time of the day, although the association was not so consistent as in the case of the heat symptoms.

Young, Myers, and Dempsey (1933), and Dempsey, Myers, Young, and Jennison (1934), have reported that also in the guinea-pig heat, occurring at intervals of 16 days, is predominantly nocturnal, that it shifts with sundown, and that when the guinea-pigs are confined in a dark room, the tendency to heat at night is lost.

d. *Starting points.* In seeking to decide whether oestrogenic substances of various kinds were actually oestrus-producing, i. e. capable of evoking mating instincts in spayed rats, the nocturnal occurrence of these instincts made it necessary to make the injections with due allowance for the length of the reaction time (abt. 24 hours) so as to produce the oestrous phenomena at night. On the basis of the observed apparent association between the oestrous phenomena and the natural day-night rhythm, the wish naturally arose to try to shift the oestrous phenomena 12 hours by artificial reversal of day-light and dark, so as to enable inspection for mating instincts to be confined to the usual working hours, still under these new conditions making the injections with due allowance for the length of the reaction time.

To keep the animals in continuous dark, seemed too unbiological for practical purposes. It might, moreover, perhaps tend to disperse the occurrence of heat over the 24 hours, as found for the guinea-pig by Dempsey, Myers, Young, and Jennison (1934), rather than to confine it to the convenient day-time. In preliminary experiments, continuous dark appeared to bring all oestrous phenomena in

the rat to a standstill. Our evidence on this point is, however, open to the objection that the numbers used were too small, and the results, therefore, inconclusive from a quantitative standpoint.

As regards the theoretical side of the problem, it appeared to us to be of considerable interest, if it would be possible, after having established the existence of the rhythm, to influence it by varying an external factor, as for instance light. Most biological phenomena so far recognized as rhythmic in mammals, have not been subject to such an experimental test, because they have been either studied in man, whose day-night habits and illumination cannot easily be reversed, or in animals, which as a rule have been killed at the investigation, thus interrupting the rhythm.

Light was chosen as the external factor to be varied, not only because of its obvious rôle in determining the natural day and night, but also because it is known to stimulate the internal secretion of the gonads irrespective of its influence on muscular activity (BISSONNETTE, 1932, and several later authors; see discussion on p. 33—36). That the nocturnal activity of forest mice can be reversed by reversal of day and night, was shown by JOHNSON (1926).

We were also prompted to make our experiments by the fact that the reproductive activity of plants is under the influence of day-length (GARNER and ALLARD, 1920; see SCHICK, 1932, MAXIMOW, 1929, or MÜLLER, 1934).

The fact that the female rats are very active at the time of the night when they usually experience the strongest symptoms of heat, and that the daily amounts of muscular activity exhibits periodic fluctuations with maxima on the days of oestrus, made it natural to expect that if the muscular

activity were shifted by reversing the periods of darkness and light, the heat symptoms would be shifted exactly to the same extent.

At first, this might appear to follow merely from the well-known nocturnal habits of the rat. The point at issue would be whether the anatomical changes were also shifted, these being from a critical point of view more directly indicative of the action of oestrin within the organism and thus of the ovarian activit .

e. Object of this paper. The object of the present investigation was thus: first, to establish definitely for the female rat that under the natural alternation of day and night not only the oestrous phenomena under direct nervous influence, the mating instincts and the spontaneous muscular activity, but also the cyclic anatomical changes of the genital tract, as recognized by the vaginal smear, are preferably confined to certain hours of the natural day-night rhythm; second, to show that all these three groups of oestrous changes, the mating instincts, the spontaneous muscular activity, and the anatomical changes, are shifted 12 hours if an artificial day-night rhythm is established by exposing the animals to light in the night and to darkness in the day-time.

A description in detail of these three groups of oestrous phenomena in the normal female rat and the technique employed in studying them, was given by one of us (HEMMINGSEN, 1933, p. 113—139 and 192—200) with due reference to previous authors. A recapitulation in brief appears to be desirable here, with a special view to minor modifications of technical details.

2. Technique in recording the oestrous phenomena of the rat.

A. The spontaneous muscular activity.

The spontaneous muscular activity is recorded by means of revolving wheels in the form of wire drums, which the rats may at will enter from a small nest box attached, and which by means of an automatic device continuously register on a kymograph the number of revolutions made in either direction, say every hour, also during the night. By a cyclometer connected to the revolving wheel the diurnal number of revolutions was also read off as a check of the continuous register on the kymograph. The cages and technical devices employed are shown in figs. 1 and 2.

The wheels and cages are constructed on the basis of the description of RICHTER and WANG (1926). The automatic, continuous register was devised in collaboration with Dr. H. C. HAGEDORN and Mr. BØRGE CLAUSEN.

B. The mating instincts.

STEINACH (1894, 1911, 1912, 1913) and SAND (1918) called much attention to the psycho-sexual phenomena in regard to experimental studies on rats and guinea pigs. As already pointed out by SAND (1918, p. 58; see also HEMMINGSEN, 1933, p. 108—109, and 119) the criteria of female heat used by STEINACH are inconclusive. SAND (1918, p. 56—58; and personal correspondence), who among his studies on the sexual characters of mammals has investigated, in extensive experimental series, as well the male as the female psycho-sexual character, also in rats, first points out that this character of course is strongest at oestrus, which in guinea pigs and rats is present essentially un-

changed from about February to about November. In the other months it decreases somewhat, without completely disappearing, but in these months it may be difficult to obtain safe responses.
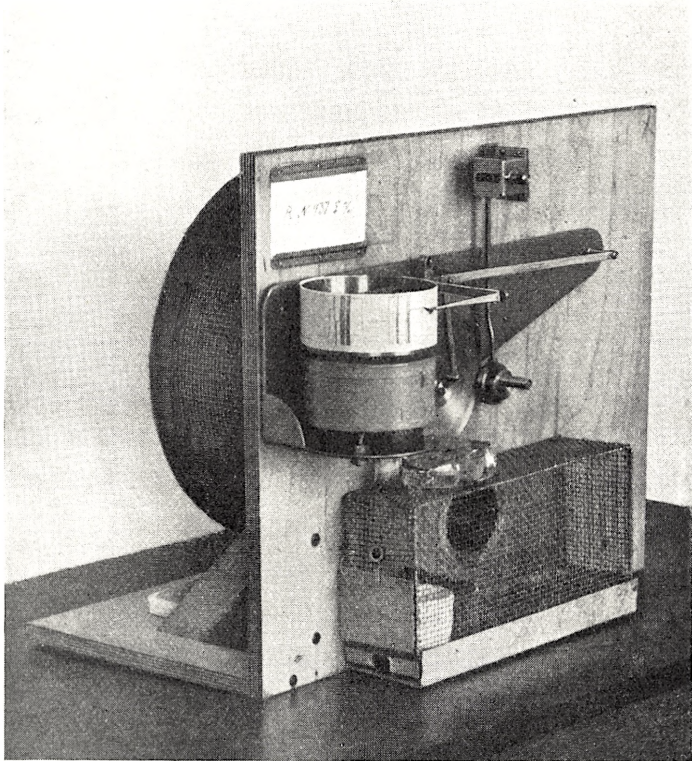
Fig. 1. Activity cage and antomatic register (cp. fig. 2).
The circular opening leads to the drum which is partly visible.

According to Sand female rats are often rather passive, but allow themselves to be pursued by males until co-pulation. Female rats in real oestrus directly approach the male and exhibit lordosis sub actu; the so-called "tail reflex" (vertical position of the tail of the female during pursuit), is sometimes observed, but not as a constant trait. Sand's experiments were made with piebald laboratory rats

of mixed origin; they were made both in day-light and by night at faint artificial light. SAND stated the conclusion (1918, p. 57—58) that the females rarely display clear symptoms of sexual desire.
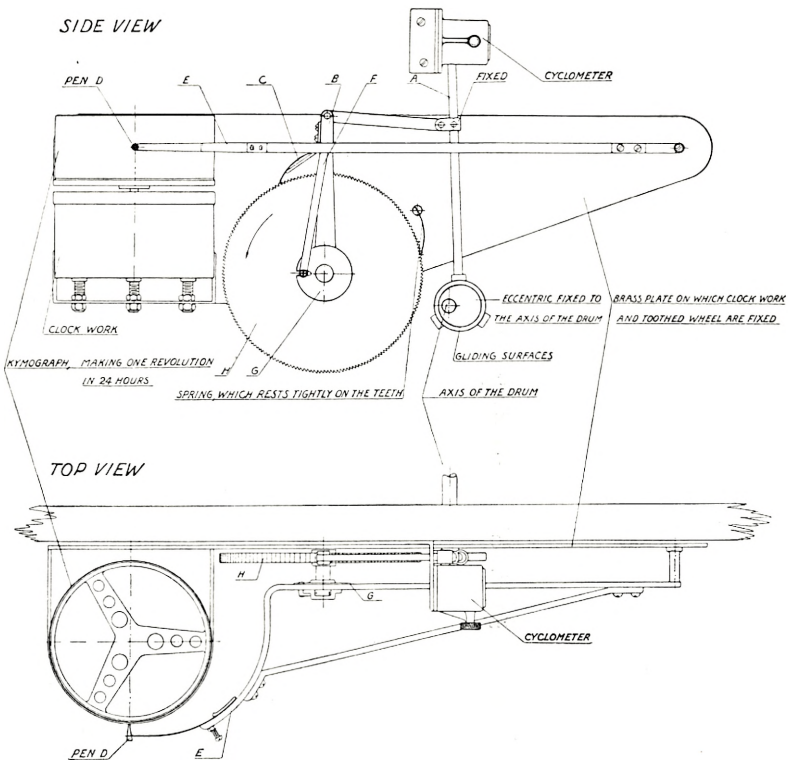
Fig. 2. Automatic register.

When the axis of the drum revolves, A moves up and down. Thereby at each revolution B, through the spring C, pushes the toothed wheel one tooth in the direction of the arrow. As there are 200 teeth, the pen D writes one peak on the kymograph for each 200 revolutions of the wheel (the distance from the lower to the upper position of the pen on the kymograph corresponding to 100 revolutions). The kymograph makes one revolution in 24 hours. A typical record is visible on the kymograph in fig. 1.

B moves from side to side behind E and F, and thus is not fixed directly to E and F. G and H are fixed together. B is penetrated by the axis of the toothed wheel between G and H.

Sand's studies on this point appear to have appeared only in Danish; readers interested in Sand's works on other points are referred to his reviews in English (1919, 1923), in French (1921), and in German (1925—32, 1933).

Later studies notably by Long and Evans (1922, p. 71—72), Stone (1922), Hemmingsen (1933), Ball (1934), and Møller-Christensen (1935), have revealed, however, that in the rat the sexual desire in the female rat is very frequently displayed and is characterized by still more reliable symptoms (a peculiar quivering of ears and body, darting hops, spontaneous lordosis) than those observed by earlier workers.

Whereas Steinach, Heinlein, and Wiesner (1925), in attempting to induce sexual receptivity in spayed rats with ovarian extracts, employed the insufficient criteria of Steinach, later Kun (1934), at Steinach's laboratory, in repeating these attempts, improved the technique so far as the observation of lordosis sub actu is concerned. Yet he did not include the above mentioned still stronger and most reliable heat symptoms (the peculiar ear quivering, darting hops, spontaneous lordosis). Kun, though on p. 316 he quotes Hemmingsen (1933), seems not to have realized that besides lordosis sub actu strong reliable symptoms (the peculiar ear quivering, darting hops) had already been produced by Hemmingsen (1933) in spayed rats with ovarian extracts.

The inability of earlier eminent workers to recognize the characteristic and frequent heat symptoms in the female rat may be due either to the inferior average quality of laboratory rats in regard to general state of health, as compared with the ideal rat strains of more recent years, or to the fact that heat in the female rat and guinea pig is

predominantly a nocturnal phenomenon, which can be observed only every fourth or fifth night.

In the present study the 12 types of mating behaviour described by HEMMINGSEN (1933, p. 119—123) have been adopted for relative measurements of the degree of sexual receptivity. The reader is referred to the photographs published in the paper quoted (figs. 1—15, p. 120—122), which illustrate the behaviour of the rats at the various oestrous stages. Since the arguments leading to the establishment of these 12 types have already been set forth in the communication referred to, it will be sufficient to refer the reader for further details to the earlier investigation.

The inspection for the degree of sexual receptivity is made as follows: The female is removed from its cage and placed in a box measuring $30 \times 30 \times 50$ cm. At inspections made during dark periods a faint electric bulb illuminates the interior of the box. Before males are introduced, it is ascertained whether the characteristic quivering of the ears (type 12 a) or spontaneous lordosis (type 12 b) is observed immediately after the female has been placed in the box, or possibly still some time after; it may be aroused, for instance, by knocking at the outside of the box. If this is not the case, the back or vaginal region of the female is tickled by one or two fingers. This must, just like the inspection for behaviour of type 12, be made in the cage and not on the table, where the animal may be distracted by the unusual surroundings. If lordosis is evoked in this way (type 11), and often this does not occur until the vaginal region is stimulated, it is, as far as our memory goes, under normal conditions practically always accompanied by quivering of the ears (we have a few observations in constant light of type 11 without quiverings). If quivering only,

but not lordosis, is evoked, the behaviour is recorded as type 10. If this stimulation produces no symptoms, a non-aggressive male is introduced. If now the female curves her back in lordosis, even though the male has not yet smelled at her vagina or attempted to mount her, the behaviour is recorded as type 9. Type 9 will probably always be accompanied by ear vibrations.

If the female only quivers her ears without exhibiting lordosis, the type is No. 8. If no symptoms are experienced, an aggressive male is introduced. If now the female quivers her ears when the male smells at her, type 7 is recorded. If there is no ear-quivering until the female is mounted, the behaviour is either of type 6, namely when lordosis is also present, or type 5, namely if only quivering and not lordosis is seen, in spite of mounting. If the response to mounting is lordosis alone, quivering being absent, the type is No. 4. If this response is a reluctant one with only slightly pronounced lordosis, it is recorded as behaviour of type 3. If neither quivering nor lordosis is produced, even after several mountings, the female is out of heat, and the behaviour is recorded as type 2. If, in spite of several attempts at mounting, the male does not succeed in achieving any mounting, the behaviour is recorded as type 1. If the males happen to make no approaches at all, the designation 0 (zero) is used, which actually merely means that the type is below 7. If the characteristic darting hops are seen, the letter d is attached to the type number.

As pointed out in the quoted paper by Hemmingsen (1933), the response of the female may vary at one and the same inspection if the above described tests are repeated, notably in the direction of increasing excitement on the part of the female, due to stimulation from the approaches

and mountings of the male. The types of sexual behaviour recorded in this paper are always based on the first response observed, by testing for the degrees in the order 12—1, as described in the preceding, and they thus form the best possible basis for comparison between the stage of sexual excitement at different hours.

It is a matter of fact that the use of the mating instincts in studies on oestrus, for instance in the experimental production of oestrus by oestrogenic compounds, represents a very rapid and convenient, let alone reliable, method of ascertaining whether a female rat is in oestrus or not.

It is, in all these respects, superior to the vaginal smear method, as described in the following paragraph. An apparent disadvantage is that in experimental studies with oestrogenic compounds larger doses are required to produce mating instincts in spayed rats than to produce vaginal cornification. Yet, it is not out of the question that a reliable assay method might be developed involving the use of the mating instincts. Another disadvantage is the nocturnal occurrence of the mating instincts. This disadvantage can, however, be disposed of, as the following pages will show.

C. *Vaginal smears.*

The vaginal samples were collected with a rustless steel-wire loop, which was heated in a flame after each sample. The smears were placed on a slide, usually several smears on each slide, and each smear received a running number. The smears were collectively fixed in 96 p. c. alcohol and stained with hematoxylin and eosin. The smears were studied and filed without a cover glass. They are still in existence.

The various stages of the oestrous cycle, as determined from vaginal smears, have been designated by the following letters:

2*

D = dioestrus or the dioestrous interval (leucocytes in the vaginal smear; some nucleated epithelial cells, or cornified non-nucleated cells, or mucus, may also be present).

P = pro-oestrus (nucleated epithelial cells in the vaginal smear, but no cornified elements, and no leuco-cytes).

P-O = transition from P to O (both nucleated epithelial cells and cornified non-nucleated epithelial cells in the vaginal smears. No leucocytes).

O = oestrus (cornified non-nucleated epithelial cells in the vaginal smear. No nucleated epithelial cells, and no leucocytes).

M = metoestrus (cornified non-nucleated epithelial cells and leucocytes).

The reasons for throwing the stages oestrus and metoestrus I, as defined by Long and Evans, together into one stage O were given in the previous communication.

The method of vaginal smears has been of paramount importance in the study of oestrin, and practically all assay work is made by means of it. On the whole it is a con-venient method, but it should be remembered that there are intermediate stages between all those mentioned, and it may, therefore, in the case of a single smear, be difficult to decide with certainty to which stage it belongs. In addition, a cornified smear does not a priori warrant the conclusion that the animal is in real oestrus (heat). A cornified vaginal smear may be found for instance during vitamin A defi-ciency even in spayed females (Evans and Bishop, 1922; Evans, 1928; Reiss and Perény, 1928), in normal females with deficient sexual activity when out of heat (Hemmingsen,

1933), and in spayed rats after frequent smear collection (WADE and DOISY, 1935; EMERY and SCHWABE, 1936). It should thus never be trusted as sole criterion.

3. Technique of illumination.

Usually the observations were started in one of the ordinary animal rooms under the normal day-night alternation of the season. After a number of oestrous cycles of 4 or 5 days, as recognized from regular periodic activity curves, had been observed, the animals, still remaining in their cages, were transferred to the light-proof experimental room, from which all day-light could be shut out. The door was opened only in entering the room for inspections, feeding, and cleaning. The daily feeding in this room was always made at the same hour irrespective of whether the light was on or off at that hour. The room was ventilated by the same system of canals as ventilate the other animal rooms.

In this room the animals were observed during some oestrous cycles under artificially established equinoctial conditions, with equal length of day and night, but still with the light period covering mainly the day time; and the dark period covering mainly the night time.

The light was automatically switched on and off at the proper hours by means of an electrical time-switch in the form of a contact-clockwork like those used on a large scale for tariff-shifting.

The artificial light used for this, and later for reversing the dark and light periods, was given from an Osram-Vitalux bulb of 500 watts, which like the sun gives a continuous spectrum with wave-lengths ranging from 270 to 4000 $\mu\mu$.

The percentage of ultraviolet rays is said to be the same as in sun-light. The rats were so placed that they could hide against the direct light, which was further somewhat tempered by a veil.

There was a tendency for the temperature of the room to rise 1°—2° C during the first hours of the periods of illumination and then to fall again during the first hours of the dark periods.

4. Preliminary observations concerning the influence of reversal of alternating light and dark 12 hour periods on the muscular activity.

a. Albino females. So much labour is imposed in carrying out vaginal inspections and mating tests at frequent intervals, that a preliminary series of experiments were made with 3 females in which only the muscular activity was studied. Since the muscular activity could be automatically registered, inspection of the animals once every 24 hours was sufficient.

Fig. 3 illustrates the results obtained with one of these rats. The others behaved much the same.

It is obvious from fig. 3 that the spontaneous muscular activity is predominantly confined to the dark periods; both under the natural alternation of day and night and after some time of exposure to light in the night and to darkness in the day-time.

That the effect of light and darkness is not an immediate and direct one, is obvious from the observation that the adjustment to the new rhythm takes place gradually, the new rhythm beginning to display its effects at a time when the effects of the old rhythm have not yet entirely vanished.

In the other two rats this overlapping of the old and the new rhythm was not marked.

The observations reveal individual differences. Thus, the maximum of muscular activity need not fall about midnight but may occur at individually different hours, as for instance in fig. 3 consistently about 20 o'clock. This individuality subsists during the gradual transition from the old to the new activity rhythm, and still after the
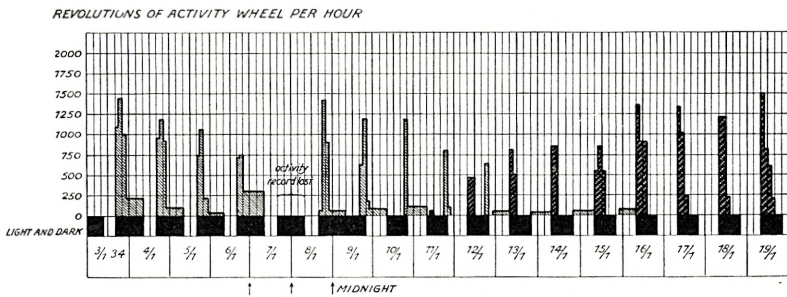
Fig. 3. Spontaneous muscular activity of a female albino rat before and after reversal of light and dark.

establishment of the new rhythm, so that after the reversal of light and dark periods the maximum of activity is at about 8 o'clock, the shift thus being just about 12 hours.

In other individuals studied later, the maximum run has been observed to shift gradually under constant day-night alternation, so that a maximum which in the beginning was before midnight, later occurred after midnight; or the converse. Instances of one maximum before and another one after one and the same midnight have also been recorded.

b. Brown females. In order to exclude the possibility that the nocturnal occurrence of the observed phenomena and the observed effect of reversing the periods of darkness and light, were dependent on the albinism of the strain of

rats used, one similar experimental series was made with three wild brown female rats. These were too wild for mating tests and vaginal smears to be made; only the muscular activity could be recorded. The effect on the activity of reversing the dark and light periods in the case of the brown rats was essentially the same as for the albinos.

An individual trait in one of these brown females before reversal was the consistent habit of displaying some, if not much, activity about noon.

c. *Albino males.* Experiments with albino males have given the same results as the experiments with albino and brown females. It should be mentioned, however, that among a number of males used for an entirely different purpose, we have observed two individuals which exhibited the main part of their activity in the natural forenoon. For reasons irrelevant in this connection, no reversal experiments could be made with these individuals, and later searching for individuals with this particular habit, was unsuccessful.

5. Main experimental series. Shifting muscular activity, sexual receptivity, and vaginal oestrous changes, 12 hours by reversal of alternating light and dark 12 hour periods.

The main experimental series included not only observations on the muscular activity, but also frequent examinations of the sexual receptivity and the vaginal smear in 6 females before and after reversal of the light and dark periods. Although two of these six rats came into constant oestrus after the reversal (continuous vaginal oestrous stages and heat of types 5—11); and though in the other four some few cycles, when considered individually, gave an unclear impression as regards the shift of the vaginal

Table 1.

The cyclic oestrous changes in the sexual receptivity of rat No. 3947 before and after reversing artificially established equinoctial dark and light periods. The various degrees of sexual receptivity are designated as described in the text (p. 17—18). The dark periods are underlined. The dark periods in which the oestrous maxima of muscular activity occur, are doubly underlined.

Date 1935	0	3	6	9	12	15	18	22	0	3	6	9	12	15	18	22	0	3	6	9	12	15	18	22	0	3	6	9	12	15	18	22
17—20/2				1								1	10	11	11					11		9	4	3				1	1	1	1	1
21—24				1	1	1	1	1				1		7	11	11				7	8	6	4		2			1		1		2
25—28				1	1	1	1	1				1	1	7	11	11				10	10	10	1	3				1	1	1	2	2
1— 4/3				1	1	1	1	2				1	1	11														1		0	1	1
5— 8				1	1	1	1	2				1	1	5		11				10	10	7	3	2				0	1	1	2	2
9—12				1	1	1	0													1	2	2	2	2				1	1	1		2
13—16				1	0	2	0	2				1	0	0	2	2				1	1	1	0	2				0	0	1	0	
17—20												0	1	1	0	2				1	0	1	0	0				0	2	0	1	0
21—24				1	0	2	0	0				1	1		0	0				1	2	2	2									
25—27						*						11	12	12	11	10				1	1	1	2	1				2	1	1	2	0
28—31				1	2	2	2	0				11	12	12	11	11				1	2	2	2									
1— 4/4				1	0	0	2	2				11	12	12	11	12					1	2	2					1		2		0
5— 8				1		2		0				11	12		12						1		1					1	1	2	2	2
9—12				1	2	2	2	2				11	12	12	12	11				0	1	1	1	2				1	1	1	1	2
13—16				1	2	1	0	2						12						1									0			
17—20					2							12									1								1			
21—24					1							12									2								1			
25—28					2							12									2										1	
29/4— 2/5				0	2	2	2	2				11	12	12	11	11				1	1	2	2	2				2	2	1	2	1
3 — 6				0	0	0	2	0				12	12	12	12						2		2					1	0	0	0	2
7 —10				1	0	0	0	2				11	12	12	12	11				2	2	0	0	0				0	0	0	0	0
11				0	0	0		0																								

*) A blank day is here inserted.

Table 2.

The cyclic oestrous changes in the vaginal smear of rat No. 3947 before and after reversing artificially established equinoctial dark and light periods. The various vaginal stages are designated as described in the text (p. 20). The dark periods are underlined. The dark periods in which the oestrous maxima of muscular activity occur, are doubly underlined.

Date 1935	0	3	6	9	12	15	18	22	0	3	6	9	12	15	18	22	0	3	6	9	12	15	18	22	0	3	6	9	12	15	18	22
17—20/2												D		P	O	O				O		O	O	M				M	D	D	D	D
21—24				D	D	D	D	D				D-P		P	O	O				O	O	O	O					D		D		D
25—28				D	D	D	D	D				P	P-O	P-O	O	O				O	O	O	O	O				M	M	D	D	D
1— 4/3				D	D	D	D	D				P	P		O													M		D	D	D
5— 8				D	D	D	D	D				D-P	P	P-O		O				O	O	O	O	M				D	D	D	D	D
9—12				D	D	D	D							D						D	D	D	D	D				D	D	D		D
13—16				D	D	D	D	D				D	D	D	D	D				D	D	D	D	D				D	D	D	D	
17—20												D	D	D	D	D				D	D	D	D	D				D	D	D	D	D
21—24				D	D	D	D	D				D	D		D	D				D		D	D									
25—27							*					P	P	P	P-O	P-O				M	D	D	D	D				D	D	D	D	D
28—31				D	D	D	D	P				P-O	O	O	O	O				O	O	O	D									
1— 4/4				D	D	D	D	P				P-O	O	O	O	O					O	M	D	D				D		D		D
5— 8				D		D		P				P-O	O		O						O		D					D	D	D	D	D
9—12				D	D	D	P-O	O				O	O	O	O	O				O	O	O	O	M				M	M	M	M	M
13—16				D	D	D	P	P-O							O					O												
17—20																																
21—24																																
25—28																																
29/4— 2/5				D	D	D	D	D				P	P-O	O	O	O				O	O	O	M	D				D	D	D	D	D
3— 6				D	D	D	D	D				D	O	O	O						O		M					D	D	D	D	D
7—10				D	D	D	D	D				P	P-O	O	O	O				O	O		O	M				M	M	M	D	D
11				D	D	D		D																								

*) A blank day is here inserted.

Table 3.

The cyclic oestrous changes in the sexual receptivity of rat No. 3950 before and after reversing artificially established equinoctial dark and light periods. The various degrees of sexual receptivity are designated as described in the text (p. 17—18). The dark periods are underlined. The dark periods in which the oestrous maxima of muscular activity occur, are doubly underlined.

Date 1935	0	3	6	9	12	15	18	22	0	3	6	9	12	15	18	22	0	3	6	9	12	15	18	22	0	3	6	9	12	15	18	22	0	3	6	9	12	15	18	22	0	3	6	9	12	15	18	22
18—21/2				0		0	1	1				0		1	11	11				10	8	9	6	6				1	1	1	1	2																
22—25				1		1	1	2				1	1	6	11			10		10		10	2					1	1	1	2	2																
26/2— 1/3				1	1	1	1	2				1	1	10	10	11				10	10	10	10	6				1	1	1	0	2																
2— 7				1	1		1													10		10	11	11				10	10	10	7	2				1	1	1		2				1	1	1	2	1
8—12				1	1	1						7	10	10	10													1	1	1	2	0				1	1	1		2								
13—18				1	1	0	1	1				1	1	5	10	11				10	10	10	10	10				0	1	1	0													1	1	1	1	1
19—23				10	6	1	1	6				12	12	12	12	10				1	2	1	0	0				1	1		0	0												1	1	1	1	
24—28												10	12	12	12	11				1	1	1	0	2				1	1	1	2	2												1	1	2	2	0
29/3— 2/4				1	5	4	0	1				12	12	12	12													1	2	1	1	1												1	0	1	1	0
3— 7					1	2	2					12		12		10				1		1		1				1	1		0													1		1		
8—12				1	1	1	1	0				12	12	12	11	11				5	1	1	1	0				1	2	7	1	2												1	1	1	2	2
13—17				1	6	6	5	5							10					1																1										1		
18—22				1									12							5																1										1		
23—27				1									12							1																1										2		
28/4— 2/5						1						11	12	12	12	11				5	1	0	1	2				0	5	1	1	1												1	1	1	2	1
3— 7				1	1	0	1	0				12	12	12	12					1		0						1	1	2	2	2												1	1	2	1	2
8—11				1	1	2	5	5				12	12	12	12	10				1	0	0	0	0				0	2	0		1												1	1	2	1	2

Table 4.

The cyclic oestrous changes in the vaginal smear of rat No. 3950 before and after reversing artificially established equinoctial dark and light periods. The various vaginal stages are designated as described in the text (p. 20). The dark periods are under-lined. The dark periods in which the oestrous maxima of muscular activity occur, are doubly underlined.

Date 1935	0	3	6	9	12	15	18	22	0	3	6	9	12	15	18	22	0	3	6	9	12	15	18	22	0	3	6	9	12	15	18	22	0	3	6	9	12	15	18	22	0	3	6	9	12	15	18	22
18—21/2				D	D	D	D					D		D	D-P	P-O				O	O	O	O	O				D	D	D	D																	
22—25				D		D	D					P	P	P-O	O	0				O		O	O					M	D	D	D	D																
26/2— 1/3				D	D	D	D	D				D	D	P-O	P-O	P-O				O	O	O	O	O				D	D	D	D	D																
2— 7				D	D	D														O		O	O					O	M	M	D					D	D	D		D				D	D	D	D	D
8—12				D	D	D						O	O	O	O							M						D	D	D	D	D				D	D	D		D								
13—18				D	D	D	D	D				D	P	P	P	P-O				O	O	O	O	M				M	D	D	D													D	D	D	D	D
19—23				O	O	O	O	O				O	O	O	O	O				D	D	D	D	D				D	D		D	D				D	D	D	D									
24—28				O	O	O	O	O												M	D	D	M	M				D	D	D	D	D				D	D	D	D	D								
29/3— 2/4				D	P	P	P-O	P-O				O	O	O	O													O	M	M	D	D				D	D	D	D	D								
3— 7				D		D	P-O	P-O				O		O		O				O		D		D				D	D		D					D		D										
8—12				D	D	D	D	P-O				P-O	P-O	O	O	O				O	M	M	M					M	M	M	M	D				D	D	D	D	D								
13—17				P	P-O	P-O	O	O						O						M																												
18—22																																																
23—27																																																
28/4— 2/5												P	P	P	P					P-O	D	D	D	D				D	D	D	D	D				D	D	D	D	D								
3— 7				D	D	D	D	D				D	P	P	P					D		D						D	D	D	D	D				D	D	D	D	D								
8—11				D	P	P	O	P-O				P-O	O	O	O	O				O	M	D	D	D				D	D	D	D																	

changes, the observations on the mating instincts, and the vaginal stages on these four as a whole, left no doubt about the positive effects of the reversal. The results obtained with two of these females are recorded in tables 1—4. The two others showed essentially the same picture[1].

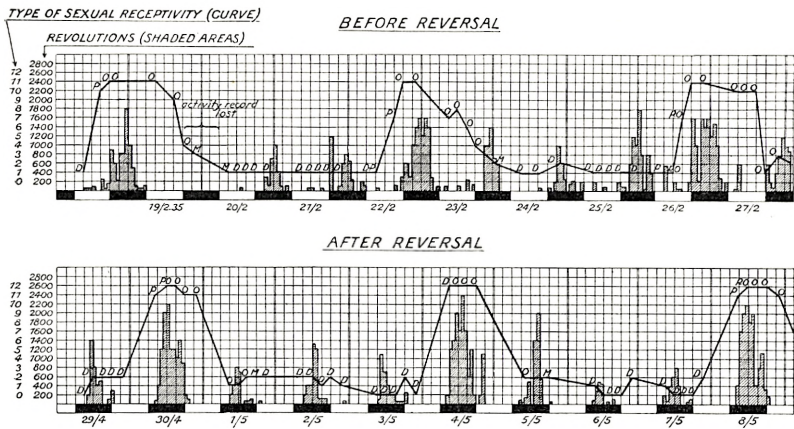A typical experiment is also illustrated in fig. 4.

Fig. 4. The relation between the vaginal oestrous stages, the degree of sexual receptivity, and the spontaneous muscular activity, in a female rat before and after reversal of darkness and light. The unit of the abscissae is 3 hours, the thick vertical lines representing midnight. The black areas below the abscissae represent the dark periods. The adjacent letters represent the stage of the vaginal smear.

In order to make the dark periods in which the oestrous maxima of muscular activity occur, fall in vertically corresponding columns in tables 1—4, allowance had to be made for the occurrence of oestrous cycles differing in length from the typical length of 4 or 5 days. Therefore, either blank days have been inserted, or extensions made to the right side of the tables. The underlinings, representing the dark

[1] Readers interested in the details can obtain the entire material through the authors or the University Library, Copenhagen.

periods, and the dates in column 1, will guide the reader on this point.

It is seen from fig. 4 that under the natural alternation of darkness and light the spontaneous activity is confined to the night, both on the days of oestrus with maximum activity, which are every fourth or fifth day, and on the other days. The figure shows also, as do the tables, that in general the vaginal stage of oestrus starts between noon and midnight, and that the sexual excitement reaches its maximum in the night. The lower part of the figure and the tables show that the time relations of the same phenomena after establishing the artificial day-night rhythm have shifted correspondingly.

With regard to the muscular activity the results of the reversal were essentially the same as in the preliminary series. All 6 females had normal oestrous activity cycles before being transferred to the experimental room on January 18th 1935. Under the equinoctial conditions in the experimental room these cycles became still more regular. The total amount of muscular activity became somewhat depressed for some few cycles after the frequent examinations recorded in tables 1—4 were started on February 18th, but then it rose again to the previous level. The total period of observation extends roughly from November 1934 to May 1935.

Beside the thorough main experimental series in point, successful reversal experiments involving less frequent examinations, have been made later with 10 other normal females. As none of these responded with continuous oestrus, the chance of obtaining successful reversals may well be assumed to be larger than the main experimental series appears to show.

It has thus been firmly established that in the female rat not only the oestrous phenomena under direct nervous influence, the spontaneous muscular activity and the mating instincts, but also the cyclic vaginal oestrous changes, are associated with certain hours of the natural day-night rhythm, the maximum of oestrus being about midnight; and that all these three groups of oestrous changes are shifted 12 hours if an artificial day-night rhythm is established by artificial day-night reversal.

6. The effect of constant light.

Four females whose oestrous phenomena had been "reversed" (not those four in the main experimental series, but some of the others in which, besides the muscular activity, only one daily mating test, in the middle of the dark period, had been recorded), and two which had been under the normal day-night alternation in an ordinary room, were all transferred to constant light in the experimental room. In all cases the effect was a marked stimulation of oestrus. The females were examined daily at 10 and 22 o'clock for types of sexual excitement above No. 10. One retained a four days oestrous rhythm, but within each oestrous cycle the sexual excitement (types 10—12) was exhibited on 2—3 days instead of only on one day. The others came into more or less constant heat (types 10—12). One of these later came into a four days oestrous rhythm, but still with prolonged heat periods. In all animals the type of behaviour was mostly No. 11. The experiment in constant light covered two months.

The muscular activity was on the average reduced. It

was in each rat still confined to certain hours, and there was a strong tendency for the activity to be displayed by all the rats during the same hours.

The period of activity was each day delayed on the average about two hours, so that after the elapse of about 12 days, and again after 24 days, it occurred again at the original time. The observations on this point cover 1 month. In our opinion this phenomenon is to be interpreted as an inherent 24 hours rhythm which, owing to the depressing influence of light, is delayed so long as it is possible for the animal to suppress it. It is as if the rat organism wishes to store the activity for a coming dark period, but as no such period comes, it cannot suppress the rhythmic 24 hours activity more than about two hours. It is thus not to be regarded as equivalent to a steady 26 hours rhythm.

The striking feature with the observations on the effect of constant light, is that light stimulates oestrus, although under an alternating day-night rhythm oestrus is predominantly nocturnal. The observations are consistent with the stimulating effect of light recorded by previous authors (Bissonnette and others, see discussion, p. 33 seq.), and add interesting evidence to support the conclusion that the normal day-night alternation is a most important factor in controlling the oestrous cycle and its associated phenomena.

7. The effect of alternating light and dark periods of 8 hours instead of 12 hours.

It follows from the relation of the oestrous phenomena to day and night, that the length of an oestrous cycle must always be a whole number of days. Average figures like

4.6, 4.8, or 5.4 days (Long and Evans, 1922, p. 45—46), never really occur. The typical, and by far most frequent, length is 4 days. It seems probable that a minimum time is required for the processes of one single oestrous cycle to be run through. Oestrous cycles of 2 and 3 days have been recorded, as well in rats with multiple ovaries (Friedman and Nice, 1930) as in otherwise normal rats, at least as recognized by vaginal smears (e. g. Hemmingsen, 1933, p. 117—118, and previous authors there cited). Thus, concurrently with the immediate object of this paper, the question naturally arises whether other rhythms may be substituted for the natural rhythm of 24 hours, and whether, with shorter rhythms, this would result in correspondingly shorter oestrous cycles than the normal of 4 days. In view of the observed vaginal cycles of 2 and 3 days, a 16 hours rhythm would not seem an unreasonable starting point in a preliminary experiment. It was decided also here in the preliminary experiments to confine the observations to the muscular activity.

A 16 hours rhythm was instituted by switching the light on and off at 8 hours intervals. With 3 adult females it appeared impracticable in this way to modify the 24 hours activity rhythm. Even after 2 months a struggle was still going on between the tendency to display the activity in the dark periods and the tendency to have one pronounced period of activity once every 24 hours (see table 5, col. 4—6).

The next experiment was to place breeding couples under the influence of such alternating 8 hour periods of light and dark, in order to prevent their offspring, even from their first origin at the time of their fertilization in their mother's body, from ever experiencing any 24 hours light-darkness rhythm. Even in these young animals the rivalling

Table 5.

The muscular activity of female rats under a 16 hours rhythm
(alternation of light and dark periods every 8 hours).

1	2	3	4	5	6	7	8	9
O'clock	Light (l) or dark (d)	Date	Number of revolutions of revolving wheels of adult female rats having been under the 16 hours rhythm from 19/1-34.			Date	Number of revolutions of revolving wheels of young female rats under the 16 hours rhythm from their birth. Their parents under 16 hours rhythm from 5/2-34.	
			Rat 3400	Rat 3244	Rat 3450		Rat 3554	Rat 3555
0— 8	l	18/3-34	0	3	0	15/4-34	303	34
8—16	d		8610	1776	1018		1278	762
16—24	l		8	0	629		287	790
0— 8	d	19	2	3	913	16		
8—16	l		1489	25	30		426	30
16—24	d		1331	670	8245		791	1087
0— 8	l	20	0	0	72	17	0	0
8—16	d		6736	2537	263		1386	358
16—24	l		8	91	204		290	355
0— 8	d	21	5	2	321	18	153	173
8—16	l		53	0	8		715	10
16—24	d		3271	2350	5967		520	568
0— 8	l	22	0	1	112	19	26	65
8—16	d		5077	478	9		369	110
16—24	l		0	7	155		1833	1107
0— 8	d	23	8	79	1921	20	175	419
8—16	l		387	9	0		984	118
16—24	d		4834	401	4712		1147	644
0— 8	l	24	2	0	41	21	780	47
8—16	d		4267	1239	3		554	836
16—24	l		4	9	73		814	92
0— 8	d	25	1	82	8580	22	154	281
8—16	l		346	61	62		71	38
16—24	d		4353	4853	933		360	191

influences of the dark, on one hand, and the endogenous 24 hours periodicity in activity, on the other hand, were still evident (see table 5, col. 8—9).

These preliminary results deterred us from making further experiments on this line. Their possible theoretical bearing will receive consideration in the discussion to follow (p. 42—45).

8. Discussion.

A. Practical side of results.

The results reported have a practical side and a theoretical side. First a few words regarding the practical side.

The present investigation has taught us that if one is interested merely in ascertaining whether or not a female rat has normal oestrous cycles, the labour involved may be reduced to merely tickling her with a finger daily at about 22—24 o'clock; or, if day and night are reversed, at about 10—12 o'clock. That this is of practical importance in all studies involving observations on the oestrous cycle in the rat, goes without saying.

So far all studies of the various purified oestrogenic compounds, α, β, γ ketohydroxyoestrin (theelin), trihydroxyoestrin (oestrinhydrate, theelol), equilinin, hippulin, etc., have been confined to the vaginal smears, more on account of the convenience of this method, than on account of the conviction it carries in ascertaining whether or not a certain compound is actually oestrus-producing. The study of the actual oestrus, the heat symptoms, the mating instincts, has been almost entirely neglected. In the case of the rat, one cause of this has been the nocturnal occurrence of the heat symptoms, which makes the studies troublesome. This communication shows how to shift the occurrence of heat to

the natural day-time. We have already made use of this practical side of our results, in demonstrating amongst other things (see the following communication) that the synthetic oestrogenic compound of Cook, Dodds, Hewett, and Lawson (1934), 9:10-dihydroxy-9:10-di-n-propyl-9:10-di-hydro-1:2:5:6-dibenzanthracene besides vaginal cornification, produces mating instincts and also enhanced muscular activity in spayed female rats.

B. Theoretical side of results.

a. Importance of shift in anatomical phenomena. As to the theoretical side, it is evident from the results that all the cyclic oestrous phenomena, not only the sexual behaviour and the spontaneous muscular activity, but also the structural vaginal changes characteristic of oestrus, are controlled by the alternation of day and night. All these three groups of oestrous phenomena are known to be controlled through the action of oestrin (see Hemmingsen, 1933, who also refers to previous authors). It seems an obvious conclusion that also the internal cyclic structural changes in the reproductive organs, both those that are controlled by oestrin, viz. the growth and distention of uterus, and the cyclic changes in the probable site of origin of oestrin, the ovary, have been shifted together with the phenomena here studied, so that the mutual correlations of all these anatomical cyclic changes become the same as under the natural alternation of day and night.

The criticism might be urged that normal oestrous periodicity of 4—5 days may be exhibited in the absence of ovarian structural changes, as shown by Parkes (1927) in X-ray-treated mice. In our rats the ovaries were excised

at different oestrous stages, after the reversal had been established for weeks. Their structure corresponded with the stage of oestrus, as recognized by the three external criteria studied. The observations were only 4 and thus much too few to be related to light and dark in the experiments. It would, however, be highly unreasonable to think that the time-relation of these structural ovarian changes, in relation to light and dark periods before reversing these periods, should have been retained after the reversal.

The experimentum crucis would be, of course, to kill rats and study their ovaries and uteri at various stages before and after reversal of day and night. This would require an extensive independent experimental series, which to us has appeared unwarranted.

b. The relation of light to oestrus according to previous authors. It was mentioned in the introduction that the influence of light on the gonads through the mediation of the anterior lobe of the pituitary has been established by previous authors.

Is the rôle of light in these previous investigations and in the observations recorded in this paper, of identical nature? They have in common the influence of light on the occurrence of oestrus; but is the resemblance more than superficial?

It is necessary before passing on to a discussion of this question, to recapitulate briefly these earlier investigations which are much concerned with the relation of light to seasonal sexual periodicity.

That light may play a rôle as regards the occurrence of oestrus, has been definitely shown by BISSONNETTE (1932), who was able to induce oestrus during the anoestrous

period of the ferret by increasing the daily light ration in the autumn. Artificial prolongation of the day time was effective. Later experiments indicated that increased light intensity would also be effective. Results of similar nature have been obtained with starlings, crows, canaries, and junco finches (Rowan; Bissonnette; Bissonnette and Wadlund; see Bissonnette, 1933), voles (Baker and Ranson, 1932a), mourning doves (Cole, 1933), ducks (Benoit, 1934, 1935 d), and Japanese pet domesticated birds (Miyazaki, 1934). That additional illumination increases winter egg-production in the domestic fowl, is a fact to which poultry farmers have long attested (cp. Baker and Ranson, 1932 a, p. 320). Prolongation of the daily light period has been practiced from ancient time in Japan to make pet birds begin to sing their mating songs as early as possible in winter (see Miayzaki, 1934)[1].

In starlings the long-waved red light is the effective part of the spectrum (see Bissonnette, 1933). According to Marshall and Bowden (1934) in the ferret heat rays and the near infra-red (wave-length 750 $\mu\mu$) are comparatively inactive. The effect begins with the red radiation (wave-length 650 $\mu\mu$) and extends to the near ultraviolet (wave-length 365 $\mu\mu$).

That, in these cases, light and not temperature is the responsible factor, is evident also from other control experiments.

According to Ludwig and Ries (1931) the activity of oestrin is augmented by red radiation, and according to Küstner (1933) and Nikolaew (1935) the time taken for an Aschheim-Zondek test for pregnancy, can be essentially shortened if the urine or the mice are exposed to red light. One feels, however, the desirability of the publication of

[1] Bissonnette's recent review (1936) adds further examples.

more experimental details in regard to this alleged direct effect of light upon the hormones.

ROWAN maintained that, at least in birds, the effect of light on sexual activity is mediated through increased muscular exercise. BISSONNETTE was able to disprove this.

For pertinent information and references the reader is referred to BISSONNETTE's reviews (1933, 1936).

The well-known effects of anterior lobe pituitary substances on the gonads, together with experiments by HILL and PARKES (1933) on ferrets and by BENOIT (1934) on ducks, lead to the assumption that the stimulating effect of light on the gonads is mediated through the anterior lobe of the pituitary gland. That in the ferret the light rays react upon some part of the head, was shown by BISSONNETTE in experiments with hooded ferrets. HILL and PARKES (1933) showed that the induction of oestrus in the anoestrous female ferret is inhibited by hypophysectomy, and BENOIT (1934), working with hooded ducks with or without perforation in the hoods for the eyes, found that light influences the development of the testes through the eyes, and that the content of gonadotropic substances, as tested by implantations into immature mice, was much larger in ducks acted upon by light than in non-illuminated control ducks.

In later experiments (BENOIT, 1935 b, c) he found that the effect through the eyeholes of hooded ducks was present also in blinded ducks, and discussed the possibility of the mediation of the effect in such animals either through direct stimulation of the cut nerve or through capability of the red rays to reach the hypophysis through the orbital "window".

It might seem at first glance an obvious working hypothesis to assume that the beginning of the mating season

in animals like the ferret, vole, starling, and others with mating seasons in the spring, is influenced by the increasing duration of day-light, whereas in other animals like some species of deer such as the red deer and fallow deer in this country and certain varieties of sheep which have their mating season in the autumn, the beginning of the mating season is determined by decreasing daily duration of light.

As the following will show, this hypothesis is too simple and should perhaps be modified so as to place more weight on the annual rhythm of light (p. 45—52).

c. Comparing the influence of light on oestrus in our experiments with previous investigations. Now, we turn again to the results of the present study, in order to compare them with the previous observations quoted.

One of the most striking experiences in this study is the observation that constant light stimulates vaginal cornification and heat, whereas under a 24 hours light-dark rhythm these phenomena are not associated with the light but with the dark periods.

It seems hardly questionable that the effect of constant light in stimulating oestrus in the rat is directly comparable with the effect of light in producing oestrus during the anoestrous period of species with a definite mating season. On the other hand, quite obviously the rôle of the light in controlling, through the day-night alternation, the oestrous cycle of the rat, on the one part, and in stimulating oestrus in the ferret, starling, duck, vole, and in the rat in constant light, on the other part, are different problems. In the rat, during the day-night rhythm, oestrus is associated with dark rather than with light. In the ferret, starling, duck, or vole, and in the rat when exposed to constant illumination, oestrus is produced by light.

The apparent paradox that additional light stimulates oestrus both in the ferret and in the rat, whereas oestrus at least in the rat is in the dark, calls for an extension of the present study to a non-nocturnal species. The difficulty is to find a small, suitable, polyoestrous, non-nocturnal form. The squirrel has been recommended to us by Professor Zoologiae AD. S. JENSEN. Squirrels, which seem to be monoestrous in our latitudes, are polyoestrous in Southern Europe and Algiers (HEAPE, 1901, p. 17; MARSHALL, 1922, p. 38) and may perhaps in captivity be made so also here. However, the question whether in non-nocturnal forms heat is actually confined to, or preferably experienced in, the light, appears to have received from zoologists but little, if any, direct study.

d. Possible mediation of the observed effects of the day-night rhythm through the anterior lobe of the pituitary. If the stimulating effect of constant light is mediated through the anterior lobe of the pituitary; is this true also with regard to the influence of the day-night rhythm on the oestrous phenomena?

The theory has been put forward (MOORE and PRICE, 1930, 1932; MOORE, 1931; BROUHA and SIMONNET, 1931; MØLLER-CHRISTENSEN, 1935) that the periodicity of oestrus arises from an interplay of the anterior pituitary and the ovary. It is assumed that the follicle-stimulating hormone of the anterior pituitary stimulates the production of oestrin, which in turn produces the oestrous phenomena. Further that oestrin inhibits the production of the follicle-stimulating hormone. Thus, the stimulus to a certain oestrous period is not applied until the inhibitory effect of the preceding oestrus upon the pituitary has vanished.

It has, in fact, been found that the content of gonadotropic

hormone of the anterior lobe of the pituitary exhibits rhythmic changes in relation to the oestrous cycle (Smith and Engle, 1929; Wolfe, 1931).

It may not be out of place, therefore, to suggest the possibility that the influence of the day-night rhythm on the oestrous phenomena of the rat may be mediated by the anterior lobe of the pituitary, and thus, that the primary effect of the reversal of light and dark may be to shift the cyclic changes in the anterior lobe.

Hill and Parkes (1934) have expressed the view that the oestrous cycle is normally due to some inherent rhythm of the anterior pituitary and occurs independently of external factors. The results of the present investigation may not be inconsistent with an inherent rhythm of the pituitary, but shows that the oestrous cycle, irrespective of whether or not it depends on a pituitary rhythm, must be dependent on at least one external factor, viz. light.

It is known from the observation of oestrous phenomena on the first two days following ovariectomy, that in the normal female rat the stimulus to oestrus operates about 24—36 hours before oestrus starts, and that it has a certain duration, perhaps about 12 hours (cp. Hemmingsen, 1933, p. 142—148). Thus the shift produced in the present study must probably mean a corresponding shift in the time-relations of this pituitary stimulus.

e. Observations on continuous oestrus. It requires notice that out of the 6 females employed in the main experimental series in which day and night were reversed, 2 came into constant heat, which lasted one month until they were killed.

In these two rats the stage of the oestrous cycle, at which the day-night reversal in the form of duplication of a night

period was undertaken, happened to coincide with the stage when the stimulus to oestrus must be assumed to have been acting; whereas in the others the reversal occurred at an earlier stage of the cycle. One could imagine that there had been a prolongation of the stimulus due to the artificial prolongation of the night when the stimulus occurred, and that this has brought the wave-like sequence of phenomena out of order. Experiments with ten females (already referred to in the preceding) which were "reversed" at the stage in question, failed to confirm this hypothesis.

In the two rats in point the strength of this constant heat showed a tendency to being higher in the dark periods than in the light. On autopsy their ovaries were found to be filled with follicles with a diameter of up to 0.6 mm. Corpora lutea were absent.

Apart from these two females, and the prolonged heat in the 6 females placed in constant light, continuous heat in female rats including the strong symptoms of sexual receptivity (above type No. 7), has been previously described by HEMMINGSEN (1933, p. 152—155), in one of the rats with transplanted ovaries, and by MØLLER-CHRISTENSEN (1935) as a consistent phenomenon in normal female rats connected in parabiosis with male or female castrates.

In MØLLER-CHRISTENSEN's experiments hyperfunction of the anterior pituitary of the castrated partner was the probable cause of the constant heat of the normal partner. In the present experiments the constant heat of the two rats exposed to "reversed" conditions may perhaps be explained as due to a stimulating effect of the artificial light on the gonads through the pituitary gland in the two most sensitive rats. This would be an effect of similar nature to the effect of light in the experiments of BISSONNETTE and in the present

experiments with constant light. The light in our experiments was on the whole probably stronger than the day-light to which rats are usually exposed.

There are other possibilities, for instance that the frequent collections of vaginal smears or the frequent sexual stimulation in the mating tests without the natural satisfaction in the form of actual insemination has a tendency to produce follicular hyperfunction. This is no probable explanation, however, as in the experiments with constant light there was no such frequent stimulations, and yet heat was prolonged with resulting continuous heat.

The absence of corpora lutea in all the cases of constant heat here discussed, and the presence of either a few large cystic or numerous smaller follicles, implies that the exhibition of continuous heat in these animals has been unaccompanied by ovulation. In some species (e. g. ferret, rabbit) heat is normally continuous until copulation takes place (MARSHALL, 1904, HEAPE, 1905; cited from PARKES, 1929, p. 53—54), leading by mediation through the pituitary (FEE and PARKES, 1929) to ovulation and formation of corpora lutea. Copulation during the continuous heat, with insertion of vaginal plugs, as well in the experiments of MØLLER-CHRISTENSEN (private communication, not stated in his book) as in the two "reversed" rats of this study (twice in the one rat, once in the other) did not lead to pseudopregnancy or pregnancy, the heat being still continuously exhibited for a long time after these copulations. Similar studies were not made with those exposed to constant light.

f. The relation of muscular activity to heat. At the inception of our studies we were inclined to expect that the culmination of heat at night would turn out to coincide

with the hour of maximum muscular activity. It will, however, be seen from tables 1—4 that high degrees of heat may start by day long before the animals have begun to display their nightly activity and continue during the subsequent light period. In addition to this, the occurrence of strong heat in constant light in the absence of enhanced muscular activity supports the contention of BISSONNETTE, in contrast to that of ROWAN (see BISSONNETTE, 1933), that the influence of light on the sexual phenomena is not mediated through an increase of the muscular activity.

g. The relative superiority of the rhythm over the single light and dark periods. There are still other observations of strong symptoms of heat and oestrous vaginal stages in the light periods. Thus, in the first days after the reversal of light and dark, the oestrous periods which would be expected to occur in the dark periods, now changed [into light periods, occur at the expected time irrespective of the fact that they are now exhibited in light. Not until about 10 days after the reversal has the new oestrous rhythm become established.

All these facts show that the direct action of darkness does not immediately and directly produce the manifestations of oestrus. But once a certain rhythm of darkness and light has become established, the oestrous phenomena centre about the middle of the dark periods. It is the day-night rhythm as such, and not the single day and night, that determines the time relation of the oestrous phenomena to the diurnal rhythm.

Observations on the effect of similar inverse illumination on diurnal rhythms in insects and plants, are analogous to those here recorded, in so far as the old rhythm asserts itself for some time after the new external rhythm of light

and dark has become established (see Bünning, 1935 a, p. 600).

h. Do rhythms of 24 hours take up an exceptional position in biology? The apparent stubbornness on the part of the rats in retaining the 24 hours rhythm of activity under a 16 hours light-dark rhythm raises the question:

To what extent have the possibilities of modification of biological rhythms connected with the alternation of night and day, been limited by the fact that in the course of at least the last million years (Quaternary period) living organisms have been affronted with practically no variations (less than 1—2 p. c.) in the time taken for the globe to make one revolution (G. H. Darwin, 1879, 1908)?

According to Beling (1929) and Wahl (1932, 1933) attempts to train bees to seek their food at definite hours (1—3 times daily) succeeded only when the rhythm was 24 hours. The biological significance of this lies in the opening at restricted hours of certain flowers which the bees visit, or diurnal rhythmic variations in the availability of pollen and nectar (Parker, 1926; Wahl, 1933; and Kleber, 1935).

Ants and termites, on the other hand, can be trained to seek their food at rhythms other than 24 hours (Grabensberger, 1933). This seems to be most easy with forms whose food does not depend on the diurnal periodicity of flowers, although their sleep is said to depend on a 24 hours rhythm even in the absence of diurnal variations in light and temperature (Müller, 1931, p. 378).

Bünning (1935 a, p. 608) has adduced evidence to show that the diurnal periodicity in emergence of *Drosophila* imagines from the puparia, as observed also by Bliss (1926), Bremer (1926), and Kalmus (1935), and known also

from other insects, is an inherent 24 hours rhythm, which is still retained after keeping this species through several generations in a 16 (8 dark—8 light) hours or 36 (18 dark—18 light) hours rhythm, or in constant faint light. Such flies kept in constant dark or faint illumination apparently lose this periodicity, but one single stimulus in the form of intense illumination for some hours is sufficient to bring about the 24 hours rhythm in emergence in spite of subsequent constant conditions.

Similar evidence of an inherent 24 hours rhythm was obtained respecting rhythmic diurnal movements in plants (BÜNNING, 1932).

Some plants can be made to exhibit other rhythms, e. g. of 12 hours, but nevertheless change spontaneously to a 24 hours rhythm when placed in constant light or dark (see JOST, 1923, p. 375—382).

There seems for plants to be some unknown internal or external factor which tends to keep them in a 24 hours rhythm (beside JOST, and BÜNNING, see STOPPEL, 1926).

All these rhythms, both those of bees and ants (GRABENSBERGER, 1934; KALMUS, 1934) and those of plants and *Drosophila* (BÜNNING, 1932, 1935; KALMUS, 1935), can be modified by influences on the metabolism. The rhythm becomes shorter if the metabolism is raised and longer if the metabolism is reduced. The question whether the changes in temperature necessary to modify the 24 hours rhythm, are always so large that this rhythm will remain unaffected under the environmental temperatures in which the respective rhythms are of biological significance, appears to deserve further investigation.

Many marine animals living in the tidal region of the sea shore have a tidal rhythm, which is retained for some time

in aquaria in the absence of any such external rhythm; even the long 14 days rhythm of neaptide and springtide may be recognized in such aquaria (see Hoffmann, 1926, p. 653—655). The daily tidal rhythms are, of course, not 24 hours rhythms, but correspond to the lunar day. Whether these animals can artificially be taught other rhythms than the tidal rhythm, is not discussed in the work referred to.

It is obvious from these examples that some of these diurnal rhythms in lower organisms are not bound to follow the 24 hours rhythm, whereas other of these rhythms are.

That in the rat a 16 hours rhythm of muscular activity cannot be established, may be due either to an inherent rest-activity rhythm of 24 hours (this to us seems the most probable) or to the impracticability of repeatedly establishing oestrous cycles essentially shorter than 4 days, so that the inherent oestrous periodicity with its maximum of muscular activity at oestrus interferes with the influence of the light-dark rhythm on the muscular activity. In the end this would mean that the oestrous periodicity is bound to obey the controlling 24 hours rhythm. Experiments with males or castrates may elucidate this point further.

Whether the various diurnal rhythms which seem to be correlated with the daily rest-activity rhythm of higher animals including man (see introduction), can be modified so as to deviate from the 24 hours rhythm, regularly through reasonably long periods of time, to us appears questionable. We have obtained from the rats the impression that there is a very strong tendency to a 24 hours rest-activity rhythm, which can be suppressed only for a limited period of time (cp. p. 28). But we would expect these various diurnal rhythms, including the rhythm in liver function, to be

susceptible to shifts, including reversals, of the daily light-dark 24 hours rhythm.

It is striking that out of the diurnal rhythms dealt with in the preceding paragraph, those known to be dependent on the alternation of day and night (bees, *Drosophila* plants, rats) can be shifted at will by shifting the determining external or diurnal light rhythms, so long as the actual length of the rhythm is maintained; whereas it seems impossible in the organisms in point to establish rhythms of lengths other than the natural.

Further, such ants as besides seeking other kinds of food also visit flowers, appear not as readily to accept rhythms different from 24 hours as forms which never seek food in flowers (Grabensberger, 1933).

We quite realize that a generalization of this statement cannot be made, and we regard the statement as a tentative suggestion, which may lead to a final scrutiny of the question in the hands of others. No doubt numerous instances can be collected of diurnal rhythms suitable for further work (examples: WELSH, 1935; YOUNG, 1935; PARK, 1935).

i. Comparing the seasonal and diurnal types of sexual rhythmicity. As already pointed out, the recurrence of short oestrous cycles in polyoestrous species like the rat or mouse, with or without a restricted mating season, is not comparable to the seasonal recurrence of mating seasons in species like the monoestrous ferret, or the polyoestrous vole.

It might appear, therefore, that the seasonal sexual periodicity of animals with long periods of sexual quiescence should be kept quite distinct from the periodic phenomena within the oestrous cycle of polyoestrous species, and that neither of these two types of periodicity could throw any light on the other.

In our opinion, however, it may be profitable to suggest, by analogy, a tentative hypothesis about the rhythmic nature of the seasonal sexual periodicity on the background of the observations on the oestrous day-night rhythm recorded in this paper.

There are some traits with the seasonal and diurnal rhythms under discussion, that appear to be of related nature, as for instance, on the one hand, the two facts that in the ferret the initial mating season changes are evident histologically already in December, before the amount of day-light has ceased to decrease (Allanson, 1932), and that in the rat the initial oestrous phenomena, both as regards anatomy and behaviour, may start already during the daily light periods; and, on the other hand, the two facts that in the ferret the onset of the mating season in the spring cannot be prevented by hooding the ferrets or keeping them in total darkness $23^1/_2$ hours per day from the end of January onwards (Hill and Parkes, 1933), and that in the rat an oestrous period, which is about to start cannot be prevented manifesting itself during an artificial light period at the time when the organism from preceding experience, would expect darkness.

In keeping the ferrets in total darkness from January onwards there was actually on the average a lag in the times of onset of oestrus, but it is evident that the effect of seasonal changes in the length of day-light on seasonal sexual periodicity, is no immediate and direct one. This applies to the ferret. In other species, e. g. the duck (Benoit, 1935 a) the effect may be direct.

Hill and Parkes (1933) infer from the experiments just quoted that the increasing day-light in the spring is not a factor influencing the normal sexual periodicity of the

ferret. The question is however, whether the annual rhythm of alternating decreasing and increasing day-lengths may not be in the end an important controlling factor for the seasonal reproductive periodicity. Nor as regards the diurnal rhythmic changes in the rat the effect of daylight is an immediate and direct one. The diurnal internal sexual rhythm will not adapt itself to the new external rhythm until this has been repeated, or so to speak reinforced, for some time.

Observations on the seasonal rhythm of various species brought to their antipodal hemisphere, appear to represent a striking analogy with the gradual adaptation of the female rat to the new light-dark rhythm, as illustrated for instance in fig. 3 of this paper.

Thus, instances are known of reindeer brought from the northern to the southern hemisphere (OLSTAD, 1930, p. 11— 12; the same mentioned by DEGERBØL, 1935, p. 26), and ponies brought from the southern to the northern hemisphere (cited from MARSHALL and BOWDEN, 1934, p. 420), which in their new locality first came on heat at the same time of the year as in their native country, i. e. at a different season; but subsequently adjusted their sexual periodicity to the new conditions.

Several other examples are known of a similar adjustment to an antipodal hemisphere. Whether it is always a "lagging" adjustment, we have not been able to ascertain; but probably it is, at least often, so. Such examples might be quoted as regards several species brought from the southern hemisphere to Zoological gardens in Europe or North America, e. g. penguins (private communication by Dr. TH. MORTENSEN), geese and black swans from Australia, sheep from South Africa (cp. MARSHALL, 1922, p. 24). Also

ferrets shift their mating season when they are brought from the northern to the southern hemisphere (Zucker-man, cited by Bissonnette, 1935).

From the following statement by Marshall (1922, p. 24, cp. also p. 45) one gets the impression (if we have not misunderstood the statement) that some observations on camels form an exceptional example in this connection: "..... whereas the occurrence of breeding in any one country or locality is closely connected with the climatic conditions and the periodicity of the seasons in that country, this rule does not hold invariably. For while the sheep in South Africa breed in April and May (the South African autumn), thus following the seasons (since sheep breed ordinarily in autumn in this country), the camels in the Zoological Gardens in London experience rut in the early spring, or at approximately the same time as the breeding season of the wild camels in Mongolia". Since London and Mongolia are both on the northern hemisphere, to us it seems that if the camels represent an exception it is one of those that test the rule.

The seasonal and diurnal periodic phenomena in point thus have in common, as far as we know, the somewhat lagging adjustment of an inherent internal sexual rhythm to an external rhythm, which in the latter case is, and in the former case may be, a rhythm of varying amounts of light.

It should be noted, that apart from the obvious fact that the seasonal sexual rhythms under consideration are not directly comparable with the day-night association of the 4 or 5 days oestrous rhythm in the rat, there seems to be one further point where the analogy between these two forms of sexual rhythm cannot be carried through: The seasonal sexual rhythm in the ferret, though it can be only slightly influenced in the form of a slight delay in the spring, can be definitely influenced in the autumn, whereas in the rat the prolongation of a dark period does not bring about

an immediate occurrence of oestrous phenomena. This difference, however, must be attributed to the fact that light and not dark stimulates oestrus in the rat, and need not affect the impression that in both groups of rhythmic phenomena we have to do with related underlying biological mechanisms: the mechanisms of adjustment to shifts in rhythmic environmental factors.

It seems not far-fetched to imagine that these mechanisms are related to the mechanisms operating in the form of conditioned reflexes, of which many types bear witness to the existence of a sort of biological clockwork of the nervous system. A similar point of view was taken with regard to the vanishing 4—5 days rhythm in spontaneous muscular activity occurring after ovariectomy or after discontinuation of periodic oestrin injections (HEMMINGSEN, 1933, p. 200—202 and 211—214).

Apart from the seasonal changes in light, seasonal changes in other ecological factors, like type and quantity of food, temperature, and rain, may undoubtedly, under certain conditions, control the breeding time, not least in tropical countries (see discussions by BISSONNETTE, 1932 b, 1936, and MARSHALL and BOWDEN, 1934).

One cannot exclude a combined influence of light with other factors like food and temperature, even in the cases that are apparently determined to a great extent by light.

In some animals light or absence of light may not play any direct rôle in stimulating oestrus. In hedgehogs (ALLANSON and DEANESLY, 1934) changes characteristic of the mating season can be produced in winter merely by keeping the animals in the laboratory. In ground squirrels (MOORE, SIMMONS, WELLS, ZALESKY, and NELSON, 1934) daily addition of light failed to produce observable effects,

whereas females kept continually for several months in cold (and darkness), with more or less normal hibernation, exhibited sexual development at any time of the year. Craig-Bennett's study on sticklebacks (1931) stresses the absence of a light effect, but shows the importance of relative changes in temperature. In field mice (Baker and Ranson, 1932 a, b, 1933) sometimes one factor seems to act, sometimes another.

But, even in animals where factors other than light, entirely or partially, determine the mating season, the influence of the seasonal rhythmic changes of such other factors, or complex of factors, may have to be considered under essentially the same aspects, only in the arguments substituting for the annual rhythm of light the annual rhythm of the complex of determining factors.

Even the mysterious lunar rhythm among marine crustacea, sea urchins, and polychætes, notably the palolo worms (see Hoffmann, 1926, p. 655—658) may have to be considered from this view-point, for instance in explaining that these worms may experience their breeding phenomena at the usual time of the year in aquaria in the apparent absence of the usual lunar influences.

Besides the observations of this study other similar observations of previous authors concerning the gradual adjustment of biological rhythms in insects and plants to variations in external rhythms, may be cited in favour of the views here advanced (see Bünning, 1935, p. 600).

The efficiency of light in causing in certain species the seasonal periodicity of reproduction, may have become evolved as an adaptation to those seasonal environmental conditions which are correlated with the amount of daylight through the seasonal light variations, in the sense that

the rhythmic day-light variations advertise in due time the organisms of the approach of the environmental conditions to which the manifestation of the whole sequence of breeding phenomena have been adapted.

The insertion of long intervals between the mating season and ovulation as in bats or a long quiescent period following the first segmentations of the fertilized ovum as in roedeer and badger, must be secondary phenomena which have been caused by gradually changing environmental conditions. No matter whether such intervals are inserted or not; and no matter what factors determine the length of the gestation period in general (for discussion see MARSHALL, 1922, p. 29—30), it is plain a priori that the mating season must be so placed that the young are produced at an auspicious time.

The annual rhythm of decreasing and increasing daily light rations, would appear to be a most reliable clockwork for any species to trust, in seeking to achieve this end.

By higher plants this clockwork is actually trusted to achieve a similar purpose. Thus, in higher plants the duration of day-light determines the time of flowering. In some plants short days favour early flowering; in others, long days, according to the natural habitat of the respective plants (GARNER and ALLARD, 1920; see MAXIMOW, 1929, SCHICK, 1932, and MÜLLER, 1934).

Likewise, according to MARCOVITCH (1924) the appearance of sexual forms in plant lice and their migrations to alternate hosts, are controlled by the length of the day.

In conclusion, we do not wish anything more than to suggest that all the apparently conflicting observations, in respect of the relation of light to seasonal sexual rhythms, may be brought under one point of view covering all the

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ground, by assuming that in the animals where light is an important determining factor there is an inherent tendency to an internal reproductive annual rhythm, and that the external seasonal light rhythm controls at what time of the year the breeding season occurs. On shifting the external rhythm there is some lag on the part of the internal rhythm in adjusting itself to the external rhythm.

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9. Summary.

1. Under the normal alternation of day and night not only the oestrous phenomena under direct nervous influence, viz. the spontaneous muscular activity, as recorded by activity cages, and the mating instincts, but also the cyclic anatomical changes of the vaginal epithelium are

preferably confined to certain hours of the natural day-night rhythm. Heat and activity are at their maximum in the dark.

2. All the three groups of oestrous phenomena mentioned, are shifted 12 hours if an artificial day-night rhythm is established by exposing the animals to light in the night and to darkness in the day-time.

3. The convenience in using, for all studies on oestrus in the rat, the reliable psychical symptoms of heat instead of the less reliable vaginal changes, is emphasized. It is an added advantage that they can be studied by day, if the animals are placed in a dark room which is illuminated at night.

4. Reversal of light and dark periods produces a shift in muscular activity not only in albino females, but also in brown females, and in males.

5. A rhythm involving the alternation of 8 hours of dark and 8 hours of light, does not abolish the 24 hours activity rhythm, even in rats kept under these artificial conditions from before birth.

6. Constant light stimulates heat and vaginal cornification, although the muscular activity is somewhat depressed.

7. An attempt is made to consider the influence of light on the seasonal sexual rhythm of some animals with a restricted mating season, on the one part, and the effect of the day-night rhythm on the oestrous cycle of the rat, on the other part, under one point of view, suggesting the theory that in both cases the fundamental fact is the somewhat lagging adjustment of an inherent internal sexual rhythm to an external rhythm of varying light rations.

(From Nordisk Insulinlaboratorium, Copenhagen, Denmark.)

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