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THE SPERM-SHEDDING RESPONSE OF MALE TOADS AND TREEFROGS AFTER THE INJECTION OF TWO TYPES OF GONADOTROPHIN

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Injection of gonadotrophin into the male British toad (*Bufo bufo*) is followed by the shedding of sperm, the response being of the normal quantal type (Frazer and Wohlzogen, 1951). The dose-response curve of the toad has now been investigated more fully with chorionic gonadotrophin, which is of the luteinizing type. At the same time the response to pregnant mare's serum gonadotrophin (of the follicle-stimulating type) was also studied to see if the two curves differed greatly in either ED50 or slope. The response of the treefrog (*Hyla arborea*) was also investigated, since it seemed possible that this smaller species might respond to smaller doses, and so be more suitable for the estimation of relatively small amounts of gonadotrophin.

The effect of chorionic gonadotrophin on the toad was also examined to see whether it altered at different times of the year under relatively standard laboratory conditions. The effect of alteration in environmental temperature was also studied. With this knowledge, the gonadotrophin excretion during pregnancy was followed in 5 cases, using a technique already developed (Frazer and Wohlzogen, 1951).

METHODS

Experimental Animals.—Toads and treefrogs were kept in an animal house at approximately 21.5° C. The experiments were normally conducted in a laboratory kept at about 20° C. The conditions during the temperature experiments are described later. In the seasonal work, the batches of toads were taken as far as possible through the summer at 6-week intervals, being used exactly one week after they had been obtained from the dealer. The interval between receipt and use was left to avoid using toads which might already have been shedding sperms. The isolation of the males from females for a week is normally sufficient to achieve this object, even when, on receipt from the breeding ponds, the cloacal urine of the males is swarming with spermatozoa. They were kept during this week in large cages containing moss and water, and fed on maggots and flies.

The toads were from the south of England, and the treefrogs were of sp. *typica* from N. Italy.

Gonadotrophins.—International Standard gonadotrophins were used for the dose-response curves, the luteinizing type from human pregnancy urine (UP) and the follicle-stimulating type from pregnant mares' serum (PMS); for the experiments on temperature, a crystalline preparation was made from pregnancy urine by the method of Claesson, Hogberg, Rosenberg, and Westman (1948). The gonadotrophin was always dissolved in distilled water; the injection volume was 1 ml.

When gonadotrophins were being estimated in pregnancy urine, the urine was filtered and injected directly. Dilutions of urine were also used, and gonadotrophin concentrates were made from samples when necessary, either because of the toxicity of the urine or because there was only a low level of gonadotrophin in it. For this purpose, the method of Scott (1940) was used, modified by the use of Universal Indicator (B.D.H.). The technique of Frazer and Wohlzogen (1951) was used for gonadotrophin estimation.

Experimental Techniques.—All injections were made into the dorsal lymph sac of toads or treefrogs whose urine was free of spermatozoa. The method has already been described, as well as the technique of sampling toad's urine (Frazer, 1950). Cloacal urine was taken 3 hr. after injection, and the presence or absence of spermatozoa noted.

At all times the amphibians were handled gently, so as to minimize struggling. If, in spite of these precautions, there was some reflux of injected fluid, the animal was rejected and another one taken. This was necessary, since the loss of one drop (0.05 ml.) represented a loss of 5% of the total given.

RESULTS

Dose-response Curves.—Tables I and II give the results with International Standard gonadotrophins, the corresponding dose-response curves being plotted in Fig. 1. Table II shows no appreciable

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TABLE I

SPERM-SHEDDING RESPONSE OF TOADS AND TREEFROGS AFTER INTERNATIONAL STANDARD GONADOTROPHIN (UP AND PMS)

Species	Gonado-trophin	Dose (i.u.)	No. Positive	No. in Group
B. bufo ..	UP	4	12	155
		6	44	130
		7·5	61	130
		9·375	67	110
	PMS	20	2	30
		22	9	30
		24	22	30
H. arborea	UP	1	20	50
		1·5	36	50
		2·5	46	50
	PMS	2·5	8	36
		3·5	12	25
		5	30	36

TABLE II

SPERM-SHEDDING RESPONSES OF MALE TOADS AT DIFFERENT SEASONS

The smallness of the groups in October and January was due to difficulty in supply. For the same reason, the January results were obtained with the survivors of the October experiments. Otherwise, fresh batches of toads were used for each experiment

Season	Number of Toads at Each Dose	Number of Toads Responding		
		6 i.u.	7·5 i.u.	9·375 i.u.
April ..	30	9	10	17
May–June	30	11	15	18
July ..	30	11	16	21
October ..	20	5	10	11
January ..	20	8	10	—

seasonal variation, so results obtained with 4 i.u. UP on toads during March and April have been included in Table I. It has thus been possible to find a characteristic curve for use with pregnancy urine throughout the year.

Gonadotrophin Content of Pregnancy Urine.—This was calculated at intervals during the pregnancy of 5 subjects (Frazer, 1954a), the equation of Fig. 1 being used to measure the 24-hour output. The method was devised to give 95% confidence limits, and these are given for a typical case in Fig. 2, where the means have been joined up to form a curve.

Effect of Temperature.—Although the findings under standard laboratory conditions had shown no significant seasonal variation in response, experiments with the crystalline preparation at varying laboratory temperatures gave:

0/12 positive at 5° C.; 28/39 positive at 16–17° C.; 39/40 positive at 22–24° C.

Further, 14 toads treated at 22° C. all showed only moderate numbers of sperms, whereas all save one

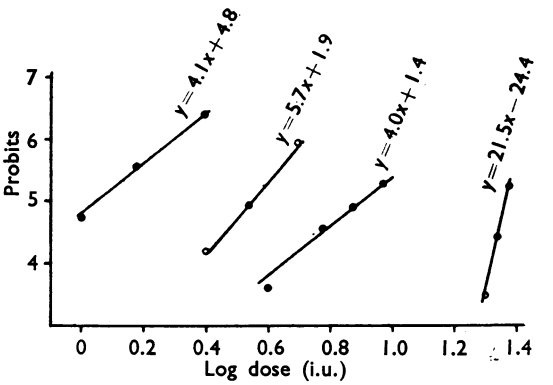

FIG. 1.—Dose-response curves for *B. bufo* (on right) and *H. arborea* (on left) when injected with gonadotrophins. The solid circles give responses to UP and the hollow circles to PMS. The formulae from which the lines were drawn are given.

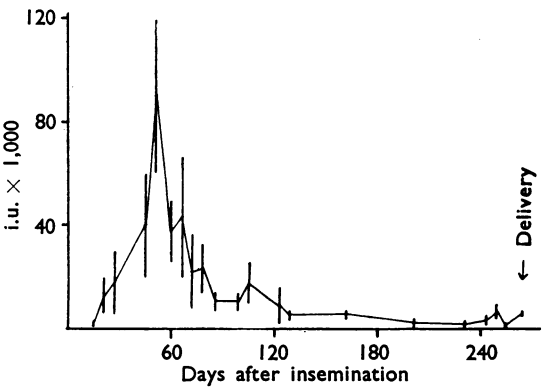

FIG. 2.—Day to day 24-hr. gonadotrophin outputs from a typical human pregnancy. The vertical lines indicate the 95% confidence limits.

of a similar group at 26° C. had numerous sperms in their urine.

Apart from the absence of sperm shedding at 5° C., keeping these toads for a further three hours at 20° C. only resulted in five positives, against a control group at 20° C. throughout, which showed 11 positives out of 12 in three hours. This slowness of response, even after the low temperature had been succeeded by the normal one, led to two further batches of 12 toads being placed at 5° C. overnight, followed by 20° C. in the morning. After an interval of 1½ hours, to allow their body temperature to return to the normal in that environment, each group was injected with the same mixture. One group was then kept at 5° C. and one at 20° C. In the next three hours, no positive response occurred in either group, but after 24 hours 5 in each group showed spermuria.

Discussion

The results given in Fig. 1 show that it is possible to obtain regular curves relating the dose of gonadotrophin to the sperm-shedding response of the toad and treefrog. They were obtained under relatively stable experimental conditions. It is noticeable that the slopes of the two curves for the response of *B. bufo* differ from one another. Yet Wohlzogen (1954), using this species, found the same slope for both types of gonadotrophin. A possible explanation may be that the two lots of toads receiving PMS were treated at different times of year; but there is at present no evidence on seasonal variation in the response to this type of gonadotrophin. Alternatively, some slight difference in the conditions of housing or of the experiments might have been responsible. Apart from this one curve, the slopes of the other three are remarkably alike, and are comparable also with that originally obtained by Winston (1952, personal communication) in *B. regularis*, where he found that the equation $y=4.04x-0.76$ applied to the first 60 toads he used. It is possible that the greater variation in his later findings may have been due to the much greater variation in the size of *B. regularis* males, which may be associated with greater variability in response: in *B. bufo*, it has been shown by Wohlzogen (1954) that light toads are more sensitive than heavy ones. This latter fact may also account for the present slope of 4.0 found for the response of the latter species to UP, which differs markedly from that originally found (Frazer and Wohlzogen, 1951). The ED50 has remained unaltered since the earlier work.

Both the present species show variation in the ED50 for their response to UP and PMS, which can be accounted for by the difference in the actual amount of each gonadotrophin defined as equivalent to an International Unit. Wohlzogen (1954) found corresponding responses in toads when 3.5 times as many International Units of PMS as of UP were used.

Comparison of the results shows that the treefrog was 7–8 times as sensitive to UP as the toad. Since the former can only be readily obtained in adequate numbers during the early summer months, this increased sensitivity is not enough to warrant its use throughout the year in place of the toad, though it may occasionally be a useful replacement.

While the work on treefrogs was taking place, Wohlzogen and Halama (1951) were performing a similar investigation with UP in Vienna, the only difference being that their treefrogs came from Eastern Austria. Comparison between the two sets of work (Frazer and Wohlzogen, 1953) showed that the slope of the regression line could be taken as common to both investigations with a value of 3.88, although there was a distinct variation between them as far as the ED50 was concerned: the Austrian treefrogs were the more sensitive. Using this value for the slope (to the nearest decimal place), together with an ED50 of 1.13 i.u., the equation for the response of treefrogs in England becomes

$$y=3.9x+4.8$$

This line is almost identical with that shown in Fig. 1.

Under the standard conditions, no seasonal variation in response was seen, but it was later shown that there was a change in threshold so slight as not to be normally perceptible. During 1952 the response to UP given three hours after a subthreshold dose was greater than if both doses were given simultaneously (Frazer, 1954b). But about the beginning of May there was a marked drop in response: during 1953 a similar falling-off was noticed (see Table III), so that it is now apparent that at the end of the toad's normal breeding season the response is slightly reduced, although this lowering of sensitivity is not detectable with the ordinary technique.

Table III

SEASONAL VARIATION IN THE RESPONSE OF *B. BUFO* TO INTERNATIONAL STANDARD CHORIONIC GONADOTROPHIN (UP), WHEN GIVEN IN TWO DOSES AT 3-HR. INTERVALS

Dose (i.u.)	Numbers Positive Against Numbers Injected	
	1 Hr. After 2nd Dose	3 Hr. After 2nd
A. Before mid-April		
4+1	0/30	5/30
4+2	8/30	24/30
4+2·5	6/11	8/11
B. After mid-April		
4+1·5	0/20	0/20
4+2	0/30	1/30
4+2·5	1/37	4/37

With the usual standard conditions, the results obtained on samples of pregnancy urine proved perfectly satisfactory and were in agreement with the findings of previous workers (Evans, Kohls, and Wonder, 1937; Loraine, 1950). The method would also be applicable to estimations of the level of serum gonadotrophin in pregnancy, although care must be taken not to use sera toxic to the toads. Allison (1954) has overcome this by the modification of injecting a smaller amount of serum mixed with hyaluronidase, and has obtained satisfactory responses. Gastineau, Albert, and Randall (1948, 1949) have shown that the renal clearance of

gonadotrophin is relatively constant, so that the serum levels will be reflected in the urinary ones.

Table II shows no variation in the response of *B. bufo* to UP during one year's work. Similar experiments were being performed on the same days by Wohlzogen, but using *Bufo viridis*. He found (1953, private communication) that the slope remained unaltered in this species, but that there was variation in ED50. His toads had been caught a week before use, but had been stored meanwhile in a cool cellar. Thus, in neither species was there variation in the slope of the line with season: yet *B. bufo* kept at 21.5° C. showed no change in ED50 whereas *B. viridis* kept cool before use showed such a change. It was possible that either the species difference or the variation in local conditions was affecting the response.

Although the response of *B. bufo* to UP has been shown not to alter with season under standard conditions, a very great difference occurred between the response at 16–18° C. and that at markedly higher or lower temperatures; and temperatures as low as 5° C., before or during the experiment, would inhibit the response normally expected at 22° C. Houssay (1947) in *Bufo arenarum* obtained a smaller response at 3° C. than at 26° C. Jeffree (1953) has reported that *B. bufo* kept in an unwarmed room showed greater sensitivity between March and August, which is again suggestive of the temperature effect reported here. These findings make it necessary to stress that any work where constancy of response is required should be carried out under standard conditions of temperature, both during and before the experiment.

Summary

1. The sperm-shedding responses of the male toad (*Bufo bufo*) and treefrog (*Hyla arborea*) to International Standard gonadotrophins in water have been studied. The dose-response curves of both species with gonadotrophins of both follicle-stimulating and luteinizing types have been plotted, and the formulae of the regression lines which fit these points worked out.

2. Under standard laboratory conditions, the curve when toads were given gonadotrophins from pregnancy urine was fitted at all seasons by the regression line of formula $y=4.0x+1.3$.

3. The response of toads to chorionic gonadotrophin varied with the temperature at which the experiment was performed.

4. It is stressed that regular responses can only be obtained under standard conditions.

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