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UNRESPONSIVENESS OF THE LEOPARD FROG *RANA PIPIENS* AS A TEST ANIMAL FOR BOVINE PREGNANCY

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The purpose of this research was to determine if the male of the common American frog *Rana pipiens* could be used as a test animal for bovine pregnancy.

Ever since Galli Mainini (1947) and Wiltberger and Miller (1948) first introduced the use of the male toad and the male frog respectively as test animals for the detection of early human pregnancy, there has been a considerable interest in the possible application of a similar technique for diagnosing early bovine pregnancy.

Cowie (1948) stated that many investigators have tried and failed to find gonadotropins in the urine and blood serum of pregnant cows by using rats, mice and rabbits as test animals, and that even ether-extracted pregnant cow's urine failed to provoke a positive reaction in female rabbits. Other investigators failed to find gonadotropins in the bovine placenta, endometrium, amniotic fluid, milk and saliva. Cowie concludes that if gonadotropins exist in the blood and urine of pregnant cows they must be in a form that cannot be detected by the usual biological methods.

Bhaduri (1950) claimed that bovine pregnancy as early as the 22nd day after breeding may be detected by using the male toad, *Bufo melanostictus*, as a test animal. This test is based on what Bhaduri calls "filter paper dialysis of hormone extraction from faeces." He injects the toad subcutaneously with an initial dose of 10 cc of this "dialysed" solution. If positive results (emission of spermatozoa) are not obtained within one hour, a second and, if necessary, a third injection of 5 cc each are made. No sperm emission after the third injection means a negative test.

Nadal and Biaggi (1950) obtained negative results when the male toads, *Bufo marinus*, were injected with pregnant cows' urine and blood serum separately. Although the dose of urine was only 5 cc some of the toads died within an hour after the injection. The cows ranged in gestation from 79 to 180 days.

PROCEDURE

In order to avoid false positive reactions caused by the injection of an excessive amount of liquid, whether any gonadotropin is present in it or not, the authors decided to inject not more than 7 cc at a time, but 3 cc and 5 cc doses were also

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tried. Giltz and Miller (1950) stated that 10 cc doses of distilled water provoked sperm emission in 18 out of 30 male *Rana pipiens* frogs tested. (Hereafter "frog" is used to mean male frog *R. pipiens*.)

To test if the amount of 7 cc would cause a false positive reaction when injected because of volume alone, four frogs were injected with 7 cc of distilled water each and four more with 7 cc of physiological saline solution each. All frogs were tested for presence of sperm in urine samples immediately before injection. All of these frogs gave negative results when tested twice at one-hour intervals after injections were made.

First test. Four non-pregnant and six pregnant cows were selected from The Ohio State University dairy herd. Pregnancy was determined by last date of service and subsequent rectal palpation by experienced members of the staff of the College of Veterinary Medicine. Urine-free fecal material was obtained manually from each cow's rectum. Eighty grams of feces from each cow were placed in individual collodion dialysis bags and each bag tied and placed in a

TABLE 1
Results of injections of dialysed fecal solutions using collodion bags

DOSE	NON-PREGNANT COWS			PREGNANT COWS		
	Reactions of Frogs			Reactions of Frogs		
	—	-+	++	—	-+	++
3 cc	4	0	0	6	0	0
5 cc	3	1	0	6	0	0
7 cc	2	0	2	6	0	0

TABLE 2
Results of injections of concentrated dialysed fecal solutions

Dose	NON-PREGNANT COWS		PREGNANT COWS	
	Reactions of Frogs		Reactions of Frogs	
	—	+	—	+
3 cc	3	1	4	2

graduated cylinder containing 130 cc of distilled water. All ten cylinders were placed in a domestic refrigerator for 24 hours. This dialysed solution was then filtered. Injections were made into six frogs for each cow, two frogs with 3 cc, two frogs with 5 cc, and two frogs with 7 cc, at one hour intervals. The reactions of the frogs to these injections of fecal solutions from non-pregnant and pregnant cows are presented in table 1.

In table 1, the double-minus sign (— —) is used when both frogs injected with the same solutions gave negative reactions. The minus-plus sign (— +) indicates one frog giving a negative reaction, the other giving a positive reaction. When the double-plus sign (+ +) is used, it indicates that both frogs gave a positive reaction.

Second test. These same solutions were then concentrated separately using the kaolin suspension method for hormone extraction. One frog per cow was injected with 3 cc of this solution. The results of this test are presented in table 2.

Table 2 shows that the injection of 3 cc of the concentrated fecal solution of

four non-pregnant cows into four frogs resulted in three negative and one positive reaction. The results of injection of fecal solutions from six pregnant cows into six frogs show four negative and two positive reactions.

Third test. Suspecting that because of its probable size the gonadotropic hormone molecule, if present in the feces, might not be able to pass through the pores of the collodion bag, it was decided to run another test using muslin bags.

For this purpose fecal samples were obtained from five non-pregnant and ten pregnant cows. In this case 50 grams of each cow's feces were placed in a muslin bag and each bag immersed in 150 cc of distilled water. Samples of these solutions for injections were taken after 24 hours and after 48 hours subsequent to the immersion of the feces-filled bags in water. Only 7 cc doses were used this time in each of two frogs per cow tested. The results of this test are presented in table 3.

Table 3 shows the results of injection of filtered fecal solutions of five non-pregnant and ten pregnant cows into frogs in 7 cc dosages. The reactions of the two injected frogs per cow are indicated in the same manner as indicated in table 1.

All frogs were tested for presence of sperm in their urine immediately before being injected and twice at one-hour intervals after injections were made.

TABLE 3
Results of injections of filtered fecal solutions using muslin bags

	NON-PREGNANT COWS						PREGNANT COWS					
	After 24-hr. filtration			After 48-hr. filtration			After 24-hr. filtration			After 48-hr. filtration		
	Reactions of Frogs			Reactions of Frogs			Reactions of Frogs			Reactions of Frogs		
	—	—+	++	—	—+	++	—	—+	++	—	—+	++
7 cc	5	0	0	5	0	0	8	1	1	8	2	0

RESULTS AND DISCUSSION

From the results of this and previous investigations concerning the use of various test animals to diagnose early bovine pregnancy, it appears that the pregnant cow does not liberate a gonadotropic hormone of placental origin in its body fluids nor in the feces, or that if the hormone is liberated it is in such a low concentration or of such a character that the various test animals, including the male frog, do not react to it.

These results do not agree with those obtained by Bhaduri (1950), who used a different genus of amphibian, *Bufo melanostictus*, as his test animal. Such a difference in the responsiveness to the gonadotropic hormone produced by the pregnant cow between these two amphibians is unexpected.

In the experiment with *Rana pipiens*, the author used a greater concentration of feces (in grams of feces per cc of water) and also dialysed the feces for a longer period than did Bhaduri. Concentration of the fecal solution as explained under the second test did not seem to have any effect on the frog reaction to it. In all cases where positive reactions were obtained, a small number of sperm were observed. None of the frogs used in this work died as a result of the injection of the fecal solution. No satisfactory reason can be given at the present for the sporadic positive reactions observed in tables 1, 2, and 3.

The data shown in the tables were treated statistically using Fisher's exact method for finding the probability in a 2×2 table when n is very small. In table 1, none of the injected fecal solutions from pregnant cows elicited a positive reaction. A complete explanation for the false positive results observed in case of non-pregnant cows is not attempted at this time.

In table 2, the deviation between the observed and expected was only 0.2 which means that the probability of deviations as great as or greater could occur through chance alone in 50 percent of the cases. With a probability of this size the deviation was considered statistically insignificant.

In table 3, it is shown that none of the non-pregnant cows gave a positive reaction. In the case of the pregnant cows, the probability of getting a deviation as great as or greater than the observed through chance alone is also of such magnitude that the deviation was considered statistically insignificant. These results support the hypothesis that there is no difference between the reactions to fecal solutions from pregnant and non-pregnant cows.

SUMMARY AND CONCLUSIONS

Fecal samples of 23 cows, 14 of them pregnant, stages of gestation ranging from 27 to 193 days, of The Ohio State University dairy herd were placed in bags, some collodion and some muslin, and submerged in distilled water for 24 and 48 hours. The dialysed solutions were injected subcutaneously in adult male frogs, *Rana pipiens*, in 3 cc, 5 cc and 7 cc doses at one-hour intervals. Also concentrated dialysed fecal solution in 3 cc doses was similarly injected into other frogs. Frogs' urine was then examined under the microscope. Urine samples showing no sperm two hours after the last injection were called negative.

Samples of two non-pregnant cows produced a positive frog reaction (spermatozoa were observed in injected frogs' urine), while most of the pregnant cows produced negative results.

No appreciable difference in the results was observed when using the collodion or muslin bag to obtain the fecal solution. Also, no difference was observed in the cases when the concentrated fecal solution was injected.

From the results of this work one or more of the following conclusions may be drawn: (1) the pregnant cow probably does not liberate any gonadotropic hormone in the feces; (2) the gonadotropic hormone in the feces of the pregnant cow, if present, must be in such a low titre that the male frog will not react to it; (3) the male frog is not a suitable test animal in diagnosing bovine pregnancy with the techniques commonly employed.

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