Hormonal Basis for Breeding Behavior in Female Frogs: Vasotocin Inhibits the Release Call of *Rana pipiens*

Abstract. *Inhibition of the release call in gravid female frogs facilitates maintenance of the male's clasp so that oviposition and spawning can occur. Arginine-8 vasotocin inhibits the release call by causing an accumulation of water and internal pressure.*

The mechanism for mammalian, reptilian, and avian receptive behavior involves pituitary gonadotrophic action on the central nervous system and ovary. This report suggests a novel mechanism in an amphibian, the leopard frog, *Rana pipiens* (*1*), by providing evidence that the amphibian pituitary hormone arginine-8 vasotocin ([Arg^8]vasotocin), plays a role in inducing breeding behavior in females.

When they are clasped by males, unreceptive female frogs emit a release call; this call is inhibited in receptive females. Noble and Aronson (*2*) have induced reproductive behavior in female frogs by injection of whole *R. pipiens* pituitary glands. These investigators have also used uninjected females, distended with water, as suitable stimuli for sexually active males. After quantifying the inhibition of the call resulting from water and ion accumulation, Diakow (*3*) suggested that the normal mechanism for inhibition might involve these factors. Since water and sodium accumulation typically follow treatment with the pituitary hormone, [Arg^8]vasotocin, the antidiuretic hormone in amphibians (*4*), this hormone was implicated in inhibition of the call.

The first of the experiments described here demonstrates that a pituitary factor plays a role in inhibiting the call. Eleven gravid leopard frogs [the median and range for the snout-vent lengths were 94 mm (84 to 102 mm)] were tested once for the release call. In this, as in all experiments, to mimic the behavior of male frogs, a manual clasp with the index finger and thumb was applied caudal to the pectoral girdle for 30 seconds, and the number of release calls was counted (*5*). After this test, five whole pituitary glands were injected intraperitoneally. Two days later, the females were manually tested for release calls and then placed with sexually active male frogs. The number (median and range) of release calls in 30 seconds of manual stimulation before pituitary injection was 36 (2 to 49) and fell to 17 (0 to 35) afterward. This was a significant decline according to the Wilcoxon matched-pairs signed ranks test ($T = 8.5$, $P < .05$) (*6*, *7*).

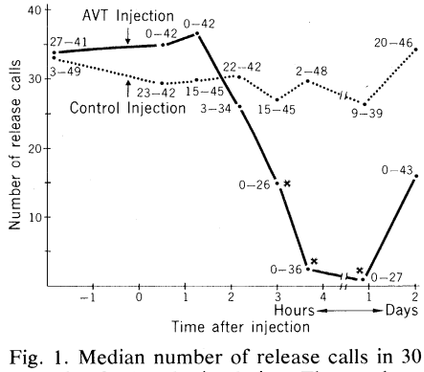

Fig. 1. Median number of release calls in 30 seconds of manual stimulation. The numbers are ranges. The x's indicate that the value for the females injected with [Arg^8]vasotocin (*AVT*) is significantly lower than that for the controls (Mann-Whitney U test, two-tailed; $P < .05$).

The second experiment indicates that [Arg^8]vasotocin inhibits the release call. Sixteen females [median and range of snout-vent length was 75 mm (71 to 80 mm)] were tested for calling manually, and the water was then removed from their tanks. Eight females received intraperitoneal injections of 20 μg of [Arg^8]vasotocin (Calbiochem) in 0.1 ml of deionized water; eight control females received 0.1 ml of the vehicle. Females were put into tap water 16 to 20 minutes after injection and were tested 30 minutes after the return to water. Thereafter, they were tested at intervals of approximately 40 minutes. The release call in the hormone-injected animals was significantly lower than that of the controls by 2 hours and 40 minutes after return to the water, and remained low the next day (Fig. 1). Recovery began within 48 hours. In contrast, no corresponding decline occurred in the release call of the control females. By 4 hours and 30 minutes after return to water, the median weight gain of the injected females was 9.2 g; the median weight loss in the controls was 0.5 g.

A third experiment shows that ion and water accumulation induced by means other than [Arg^8]vasotocin injection inhibit the release call; and on the basis of this experiment it is inferred that [Arg^8]-vasotocin can inhibit the release call by causing ion and water accumulation. Frogs placed in Ringer solution imbibe fluid and become hyperionized because of the skin's active transport of sodium (*8*). Conversely, frogs placed in deionized water lose ions (*9*). Fifty-one females [median and range of snout-vent length was 71 mm (67 to 77 mm)] were tested on the day after arrival in the laboratory. Before this test, they were housed in groups with no water added to their tanks. After this manual test, 20 females were placed in tap water, 16 in deionized water, and 15 in Ringer solution; all were tested 28 hours later. The release call was inhibited only in the group in Ringer solution (Table 1).

An attempt to differentiate ion and water accumulation as the inhibitor of the release call indicated that water accumulation unaccompanied by ion accumulation is sufficient to inhibit the call. Seventeen females [median and range snout-vent length was 84 mm (72 to 93 mm)] were tested three times at hourly intervals. The cloacas were closed surgically to prevent water loss by urination (*3*). Then, nine of these subjects were placed in individual jars containing 1800 ml of deionized water and eight were placed in 1800 ml of tap water. All were tested 24 hours later. Before the animals were put into the solutions, the resistance (*10*) of the tap water was 40 kilohms (40 to 45 kilohms); that of the deionized water, 900 kilohms (700 to 1000 kilohms). After the animals lived in the solutions for 23 hours, the resistances were 38 kilohms and 80 kilohms, respectively. Clearly the animals in deionized water lost electrolytes. Despite this, the release call declined as it did in the group housed in tap water (Table 1).

Although water accumulation might be thought to exert its effect by changing osmotic concentration or by causing a mechanical pressure that distends the body, there is evidence supporting the latter. Eight females were tested while housed in a humid, but not wet environment

Table 1. Median and range number of release calls.

Treatment or condition	Before	After
Females with open cloacas		
Ringer	40 (5 to 52)	28 (9 to 63)*
Deionized water	39 (7 to 52)	42 (14 to 53)
Tap water	42 (6 to 54)	43 (21 to 62)
Females with ligatured cloacas		
Tap water	27 (19 to 50)	5 (0 to 23)†
Deionized water	25 (16 to 41)	4 (0 to 24)†
Females with balloon implants		
Before implant	25 (18 to 40)	26 (21 to 38)
After implant	27 (24 to 42)‡	0 (0 to 0)§

*Significantly different from other treatment groups ($P < .05$, Mann-Whitney U test, two-tailed). †Significantly different before and after treatment; $P < .05$, Wilcoxon test, two-tailed. ‡Uninflated. §Inflated.

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(*11*). A balloon was implanted in each subject intraperitoneally through a midventral incision, and the end of the balloon was drawn through the cloaca to the outside. The balloons of half the group were slightly inflated; all females were tested 16 to 18 hours later. Before implant the groups were similar, but inhibition of the release call occurred only in the distended females (Table 1). The release call returned within minutes of deflation.

The above experiments suggest that in *R. pipiens* one mechanism for pituitary regulation of breeding behavior involves the ability of [Arg^8]vasotocin to cause water accumulation and subsequent internal pressure. This is the first time this relationship has been described, and more work is necessary to test its generality (*12*). This mechanism has ecological relevance, since water is obligatory for amphibian reproduction, and moisture is often the most important trigger of reproductive behavior (*13*).

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References and Notes

1. Frogs were obtained from Ward's Natural Science Establishment or from Mumley, Vermont; all were of northern origin. There is some uncertainty about the actual species provided when *Rana pipiens* are obtained from a central supply house. In order to minimize the possibility that differences between groups within an experiment are due to different species in each group, all animals in any one experiment were from the same shipment.
2. G. K. Noble and L. R. Aronson, *Bull. Am. Mus. Nat. Hist.* **80**, 127 (1942).
3. C. Diakow, *Physiol. Behav.* **19**, 607 (1977).
4. W. H. Sawyer, *Endocrinology* **75**, 981 (1964).
5. The manual stimulation method has been described (*3*), and it was shown that the results of this method parallel those obtained when male frogs are used to test the females.
6. The median latency to oviposition from the start of amplexus (clasping by males) was 2 hours 40 minutes, and ranged from 13 minutes to more than 9 hours.
7. In all experiments, frogs were housed on receipt in community tanks containing a dilute solution of Agristrep (streptomycin sulfate). When the frogs were introduced to the experiment, they were housed in pairs (first two experiments), groups (third experiment), or singly (last two experiments) in water without Agristrep. The laboratory temperature was usually 21° to 23°C, and the overhead fluorescent lights were kept on for 14 hours per day.
8. N. Mayer, *Comp. Biochem. Physiol.* **29**, 27 (1969).
9. A. Krogh, *Osmotic Regulation in Aquatic Animals* (Cambridge Univ. Press, Cambridge, 1939).
10. Resistance was measured through two electrodes 8 mm apart and 8 mm deep in solution.
11. Each frog was placed with a damp paper towel in a loosely covered jar.
12. W. Russell [*Behaviour* **7**, 113 (1954)] induced breeding behavior in a totally aquatic frog, *Xenopus laevis*, with chorionic gonadotropin.
13. S. N. Salthe and J. S. Mecham, *Physiology of the Amphibia*, B. Lofts, Ed. (Academic Press, New York, 1974), vol. 2.
14. I thank B. Colodne who assisted during the experiment with [Arg^8]vasotocin, Drs. H. Jervis and J. W. Lazar for discussion of these experiments as they were in progress, and Drs. E. Brodie, H. Grob, D. Jones, and S. Smith for comments on the manuscript.

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Sustained Release of Alcohol: Subcutaneous Silastic Implants in Mice

Abstract. *A sustained-release device for use in ethanol dependence studies in mice is described. The Silastic device, dubbed SERT (sustained ethanol release tube), holds 0.35 milliliter of 95 percent ethanol (by volume) and is implanted under the skin of the back where it releases ethanol for up to 12 hours, with no observable tissue damage. The device may be adaptable to the release of other volatile liquids or drugs, in other animals.*

Studies on the mechanisms of tolerance and dependence produced by drugs of abuse, including alcohol (ethanol), are greatly facilitated by animal models of drug intake (*1*). Many reports have been devoted to defining, discovering, and utilizing animal models of alcoholism (*2*), and the cited criteria for a useful "animal model of alcoholism" vary from author to author (*1, 3*). Although an oral route of administration would be ideal for matching the human condition of alcoholism, the view has also been taken that the route of administration is irrelevant to research examining the pharmacology of ethanol dependence and tolerance (*4*). In addition, it is impractical and tedious to administer unpalatable drugs repetitively by mouth in large samples of animals. To this end, numerous sustained-release forms of abused drugs have been reported, including morphine (*5*), pentobarbital (*6*), and amphetamine (*7*). To our knowledge, however, no sustained release forms of ethanol have been developed.

Working under the hypotheses that maintenance of continuous intoxication is the key to producing physical dependence (*4*), and that tolerance and physical dependence on ethanol can be produced by its sustained release from an implantable subcutaneous silastic tube in mice, we have developed a sustained ethanol release tube (SERT) for use in mice and other animals. The standard device is 60 mm in length and consists of Dow Corning medical grade Silastic tubing (3.35 mm inner diameter; 4.65 mm outer diameter). The SERT is sealed at one end with a siloxane polymer, closed at the other end with a glass stopper (Fig. 1), and holds 0.35 ml of liquid. The pretied neck prevents the stopper from being inserted too far and maintains the SERT in the correct position. The SERT is implanted by aseptic techniques under the skin of the back so that the sealed end is near the tail and the stoppered end protrudes through the skin at the neck of the mouse, between the ears (*8*).

We have studied the release rates of various ethanol concentrations from the SERT implants in beakers of distilled water and saline, at both room temperature and body temperature, and found that the release rates in vitro are proportional to the concentration of ethanol inside the tube (data not shown). The release rate in vivo (*9*) is faster than that in vitro, presumably in part because the ethanol that is released in the mouse is carried away by the circulation and metabolized by the liver. Although release of 95 percent (by volume) ethanol from the tube is not linear, the mean release rate in vivo from the standard SERT over 12 hours has been calculated to be 760 mg per kilogram of body weight per hour (18.2 g kg^{-1} day^{-1}), which is only slightly greater than the normal rate of metabolism of ethanol in the mouse [550 mg kg^{-1} hour^{-1} (*10*)]. We have found, furthermore, that concentrations of alcohol in the blood of implanted mice vary significantly, because of different rates of metabolism within the strain (*11*). We speculate that variable ethanol release rates from the SERT may thus occur. It is therefore desirable to screen the mice to eliminate those with very high and very low rates of alcohol metabolism (*12*). This is only for convenience, however; all animals can be used if care is taken to give each animal supplemental (intraperitoneal) injections of alcohol dependent upon its behavioral intoxication.

The rate of release of 95 percent ethanol from the SERT is ideal for maintaining blood concentrations produced by an initial intraperitoneal injection of ethanol (Fig. 2). Thus a "high" dose of 3.5 g/kg injected intraperitoneally will be maintained in a mouse with an implanted SERT until death occurs, whereas the same high dose injected into a mouse with no SERT will produce a peak blood alcohol concentration of 425 mg/dl (Fig. 2) plus ataxia or hypnosis lasting for approximately 2 hours (data not shown). This intraperitoneal dose of 3.5 g/kg is thus too high to be used in conjunction with the SERT filled with ethanol. A "low" dose of 1.0 g/kg injected intraperitoneally is adequate for producing tolerance and physical dependence with the SERT, and will initiate a blood alcohol concentration of approximately 200 mg/dl which is maintained for greater than 12 hours with only slight decrement

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