NO sexual behaviour in newts

SIR — The interaction of nitric oxide synthase with L-arginine generates nitric oxide and L-citrulline[1]. Nitric oxide exhibits many regulatory effects in many tissues and systems[2–4], including emotion-regulating brain regions[5–7]. Nitric oxide regulates sexual behaviour in mammals[8,9], but nothing is known about its effects in lower vertebrates. Here we show that nitric oxide has a role in male courtship behaviour but not in females of the urodele crested newt (*Triturus carnifex*).

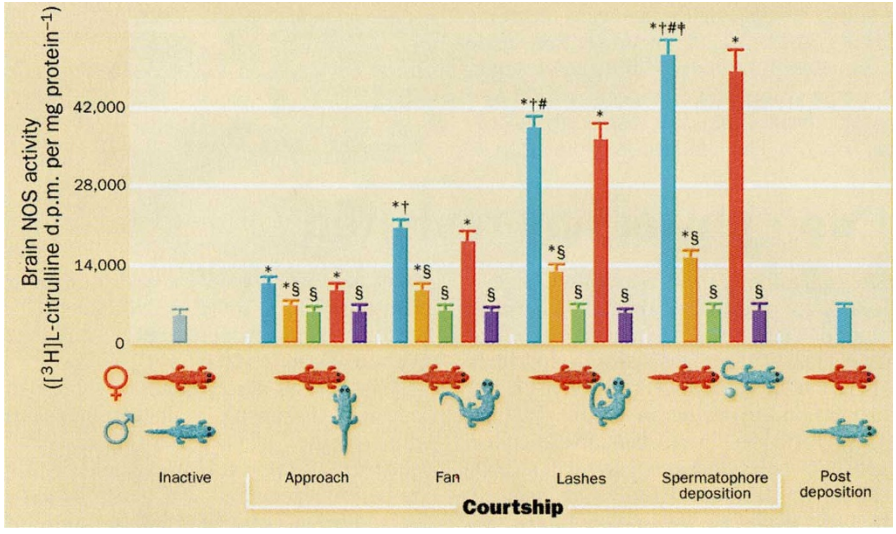
This species is a good model because it shows a complex, peculiar courtship[10–12]. The male is the active partner during courtship, which is in four phases: (1) approach, in which the male sniffs the female's head; (2) fan, in which the male slowly beats its tail towards the female's head; (3) lashes, in which the male's tail hits the female's head; and (4) deposition, in which the male shows his tail tip, the female touches his tail, he deposits the spermatophore, and she picks the spermatophore up with her cloaca. Males do not always complete courtship; in the case of females, receptive individuals remain immobile and close to the partner until spermatophore deposition, whereas non-receptive ones move away from the courting male.

We captured newts during the reproductive period, put one male and one female together in aquaria and observed the males' courtship. We divided the males into experimental groups: inactive (males which in the presence of a female did not exhibit courtship for at least 6 h), approach, fan, lashes, deposition, post-deposition (15 min after), male interruption (0 and 15 min after male courtship interruption), and female interruption (0 and 15 min after female moving away). We determined nitric oxide (NO) synthase (NOS) activity *in vitro* in the brain by the methods described in the figure legend.

Inactive males had the lowest values of brain NOS. The activity of this enzyme progressively and significantly increased during the phases of courtship, reaching a maximum during spermatophore deposition. Males killed 15 min after deposition had brain NOS levels as low as those of inactive males. Brain NOS values in males that interrupted courtship were significantly lower than those of courting males. Males whose partner had just moved away had brain NOS values similar to those of males with receptive females, but 15 min after interruption, these values had decreased to those of inactive newts. Female brain NOS activity did not change in any experimental group.

Thus there is a causal relationship between male sexual behaviour and brain NO, in agreement with observations in male mice, which exhibit inappropriate sexual behaviour and increased aggressiveness on disruption of neuronal NOS[9]. In our male newts, reproductive success is characterized by high brain NOS activity which progressively increases over the courtship various phases, just the opposite of inactive newts, which have the lowest brain NOS activity. The passage from one phase to the next seems to require a brain NO 'threshold' value to be reached. This suggestion is supported by observations that the males which interrupt courtship show a NOS activity significantly lower with respect both to those which completed the courtship phase and to those whose female partner moved away.

Female 'receptivity' and 'non-receptivity' do not depend on brain NO; in fact, NOS did not change either when the female remained close to the partner or when she moved away. Unlike males, these results in newts are not in agreement with data from female rats[8]. Many external and internal stimuli influence courtship behaviour. In *T. carnifex*, NO is probably an internal cerebral stimulus, suggesting that its involvement in reproductive behaviour could be conserved throughout vertebrate evolution.



Brain NOS activity values of male *T. carnifex* during sexual behaviour. Five males in each experimental group were killed by decapitation. NOS activity was determined in the brains by monitoring the conversion of [^{3}H]L-arginine into [^{3}H]L-citrulline, with a modified method previously described[13]. Each brain was weighed, homogenized in 1 ml cold fresh homogenating buffer (50 mM Tris, 1 mM EDTA, 1 mM EGTA, pH 7,4), and centrifuged at 20,000*g* for 60 min at 4 °C. Supernatant (25 µl) and incubation buffer (100 µl; 1.5 mM NADPH, 1 mM $CaCl_2$) containing 150,000 d.p.m. [^{3}H]L-arginine were added to the incubation tube. After 30 min, the enzymatic reaction was stopped by addition of 2 ml blocking buffer (20 mM HEPES, 2 mM EDTA, pH 5.5). The mixture was applied to a pre-equilibrated column (20 mM sodium acetate, 2 mM EDTA, 0.2 mM EGTA, pH 5.5) containing 1 ml Dowex AG50W-X8, and the material eluted with 2 ml water. [^{3}H]L-Citrulline was quantified in a liquid scintillation system. Additional determinations were performed in the presence of excess NOS inhibitor (L-NAME) to verify the specificity of the assay for production of [^{3}H]L-citrulline by NOS. Each mean refers to five determinations ± s.d. One-way analysis of variance $F_{[21,88]}$: 12.34, P <0.01. pale blue box, inactive; blue box, complete courtship; orange, immediately after courtship interrupted by male; green box, 15 min after courship interrupted by male; red box, immediately after courtship interrupted by female; purple box, 15 min after courtship interrupted by female. Symbols: Duncan's multiple range test: * P <0.01 versus 'inactive' and 'post-deposition'; † P <0.01 iversus 'approach'; # P <0.01 versus 'fan'; ǂ P<0.01 versus 'lashes'; § P <0.01 versus same phase of complete courtship.

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1. Nathan, C. & Xie, Q.-w. *Cell* **78,** 915–918 (1994).
2. Xie, Q.-w. *et al. Science* **256,** 225–228 (1992).
3. McCall, T. & Hassid, A. *Trends pharmac. Sci.* **13,** 1–6 (1992).
4. Bredt, D. S. & Snyder, S. H. *Neuron* **8,** 3–11 (1992).
5. Bredt, D. S. *et al. Neuron* **7,** 615–624 (1991).
6. Vincent, S. R. & Kimura, H. *Neuroscience* **4,** 755-784 (1992).
7. Rodrigo, J. *et al. Phil. Trans. R. Soc.* B**345,** 175–221 (1994).
8. Mani, S. K. *et al. Proc. natn. Acad. Sci. U.S.A.* **91,** 6468–6472 (1994).
9. Nelson, R. J. *et al. Nature* **378,** 383–386 (1995).
10. Halliday, T. R. in *The Reproductive Biology of Amphibians* (eds Douglas, D. H. & Guttman, S. I.) 185–232 (Plenum, New York, 1977).
11. Zerani, M. *et al. Horm. Behav.* **26,** 56–61 (1992).
12. Andreoletti, G. E. *et al. Horm. Behav.* **17,** 103–110 (1983).
13. Bush, P. A. *et al. Biochem. biophys. Res. Commun.* **185,** 960–966 (1992).