*Journal of Chemical Ecology, Vol. 16, No. 12, 1990*

# EFFECTS OF UREA ANALOGS ON EGG HATCHING AND MOVEMENT OF UNHATCHED LARVAE OF MONOGENEAN PARASITE *Acanthocotyle lobianchi* FROM SKIN OF *Raja montagui*

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(Received May 23, 1990; accepted July 30, 1990)

**Abstract**—Rapid hatching in the monogenean parasite *Acanthocotyle lobianchi* from the skin of *Raja montagui* is stimulated by urea. Structurally similar to the urea molecule, the following analogs of urea provide amino groups, carboxyl groups, or combinations of these, but fail to stimulate hatching at concentrations of 1 mM in seawater: methylurea (MU); 1,3-dimethylurea (DU); 1,1,3,3-tetramethylurea (TMU); thiourea (TU); 1,1,3,3-tetramethyl-2-thiourea (TMTU); and 1-phenyl-2-thiourea (PTU). All of these analogs except PTU elicit movements of unhatched larvae, and posttreatment of the eggs with urea showed that the ability to hatch is not impaired by initial treatment with any of the urea analogs. Thus the larval chemoreceptor that initiates hatching appears to be highly specific for the urea molecule.

**Key Words**—*Acanthocotyle lobianchi*, monogenean, *Raja montagui*, elasmobranch, parasite eggs, oncomiracidia, hatching factor, urea, urea analogs, chemoreceptors.

## INTRODUCTION

Fully embryonated eggs of the monogenean skin parasite *Acanthocotyle lobianchi* rarely hatch when incubated in seawater, and larvae within the eggs may

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0098-0331/90/1200-3523$06.00/0 

remain alive for up to 83 days at 13°C (Macdonald, 1974). However, mucus from the skin of the host (*Raja* spp.) induces rapid hatching (within 2–4 sec). Kearn and Macdonald (1976) found that seawater containing analytical grade urea, at concentrations similar to those in ray skin mucus, also stimulates hatching in *A. lobianchi*. Their finding that the stimulatory effect of ray skin mucus is destroyed by incubation with urease and restored by the addition of urea crystals, indicates that urea is an important hatching factor derived from the host. Factors such as mechanical disturbance and reductions in light intensity, which are known to stimulate hatching in other monogeneans (see Whittington and Kearn, 1988, 1989), fail to stimulate hatching in *A. lobianchi* (see Macdonald, 1974), and the apparent dependence of this parasite on a single hatching stimulant provides a useful experimental system for the investigation of chemoperception. As a starting point, it was decided to test the effectiveness of commercially available analogs of urea as hatching stimulants for *A. lobianchi*, in an attempt to throw light on the molecular interaction between the stimulant and the sensory receptors of the oncomiracidium.

## METHODS AND MATERIALS

*Collection and Maintenance of Eggs.* Living adult specimens of *Acanthocotyle lobianchi* were collected from the skin of freshly landed *Raja montagui* caught by the research vessels of the Marine Biological Association of the United Kingdom at Plymouth. On some occasions, living, egg-laying parasites from *R. montagui* were sent to Norwich in vacuum flasks of cooled seawater. Groups of 5–15 adult parasites were maintained in covered glass crystallizing vessels (5 cm diameter) containing about 30 ml of Plymouth seawater (PSW) filtered through two sheets of Whatman No. 1 filter paper to remove marine organisms that might be a source of urea. The PSW was changed each day. Eggs were collected and incubated in crystallizing vessels following a procedure similar to that used by Macdonald (1974) and Kearn and Macdonald (1976) in previous studies on *A. lobianchi* eggs. Briefly, eggs were kept at 13–14°C and exposed to 12-hr periods of alternating dim blue light and darkness (LD 12 : 12; light on, 0930 hr; light off, 2130 hr) selected to simulate conditions on the seabed at depths where rays are found (Whittington and Kearn, 1986). The PSW was changed daily during incubation and, at the same time, the eggs were examined for signs of embryonation and hatching.

*Hatching Tests with Urea analogs.* The analogs of urea chosen for testing were: methylurea (MU); 1,3-dimethylurea (DU); 1,1,3,3-tetramethylurea (TMU); thiourea (TU); 1,1,3,3-tetramethyl-2-thiourea (TMTU); and 1-phenyl-2-thiourea (PTU). The chemical structures of these substances and that of urea are shown in Figure 1. MU, DU, TMTU, and PTU were obtained from Sigma

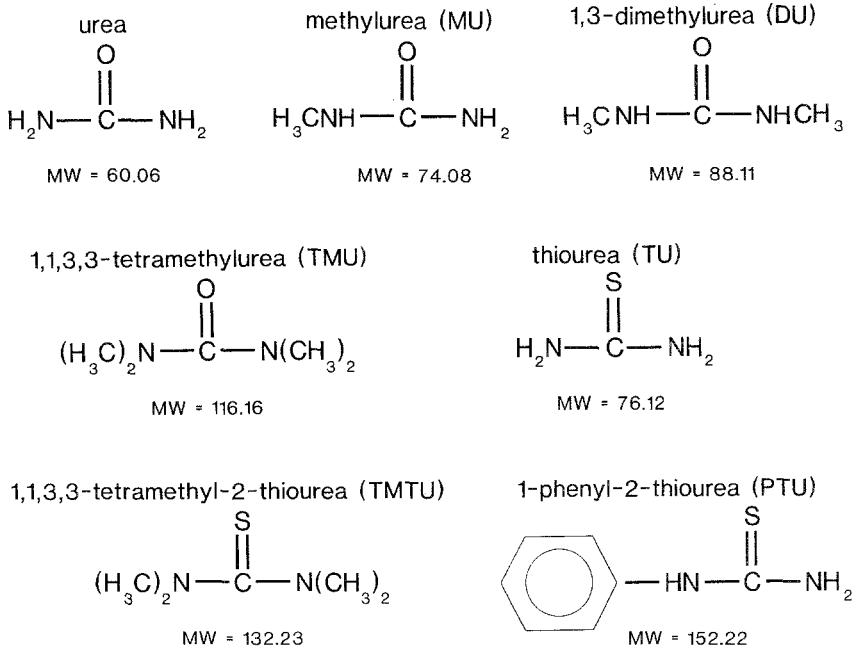


FIG. 1. The chemical structures of urea and of the urea analogs used in hatching studies on eggs of *Acanthocotyle lobianchi*. The molecular weight (MW) of each chemical is shown beneath each structure.

Chemical Co. and Analar urea (analytical grade crystals, minimum assay—99.5% urea; see below), TMU, and TU from BDH Chemicals. A 1 mM solution was chosen for each urea analog tested because a 1 mM solution of urea in seawater readily hatches *A. lobianchi* eggs and estimates of the urea concentration in mucus from the ventral skin of *Raja clavata* range from 0.8 to 1.5 mM (Kearn and Macdonald, 1976). Before use, each test solution made up in PSW was allowed to reach the same temperature as the PSW containing the eggs.

Three experiments were performed with each analog using eggs containing fully developed larvae of three different ages, namely 27, 46, and 75 days (eggs reach full development after about 15 days at 13°C, according to Macdonald, 1974). Each experiment was carried out by transferring 5–20 eggs of the appropriate age to about 10 ml of the test solution in a Syracuse watch glass. The transfer was carried out using a chemically clean pipet drawn fine enough to minimize the amount of seawater transferred with the eggs. This procedure was performed during the 12-hr period of illumination on the stage of a stereomi-

croscope with dim illumination from below. Eggs were observed continuously for 15 min for signs of either larval movement or hatching and were then removed from the test solution and transferred for posttreatment to a second watch glass containing approximately 10 ml of a 1 mM solution of Analar urea in PSW. Posttreated eggs then were observed for 15 min. With each experiment, controls were treated first for 15 min with PSW and then posttreated for 15 min with a 1 mM solution of Analar urea in PSW.

## RESULTS

No hatching occurred when eggs containing fully developed and living larvae were incubated in clean PSW for up to 75 days and no hatching occurred as a result of treatment of fully developed eggs aged between 27 and 75 days with any of the urea analogs (Table 1, experiments 1–18). Larval movements were observed in some eggs aged 27, 46, and 75 days when treated with MU, DU, TMU, TU, and TMTU (Table 1, experiments 1–3, 4–6, 7–9, 10–12, and 13–15 respectively). No larval movements were seen in any of the eggs treated with PTU (Table 1, experiments 16–18) or in the eggs in the control batches treated initially with PSW. When test and control batches were posttreated with a 1 mM solution of Analar urea in PSW, between 42.9 and 100% of the eggs tested in each batch hatched within 5 sec. No further hatching occurred during the rest of the posttreatment period, but in the unhatched eggs, irrespective of age and previous treatment, movements of larvae were observed. Posttreatment of 75-day-old eggs with urea, after initial treatment with MU, DU, TMU, TU (Table 1, experiments 3, 6, 9, 12) and PSW (control), elicited 100% hatching, but this was not the case when 75-day-old eggs were posttreated with urea after initial treatment with TMTU and PTU. With TMTU (experiment 15), 71.4% of the eggs hatched and with PTU (experiment 18) 42.9% hatched.

## DISCUSSION

As pointed out by Kearn and Macdonald (1976), it seems likely that the small urea molecule diffuses readily through the eggshell (or through the opercular seal) of *Acanthocotyle lobianchi*. Presumably the urea molecule binds to superficial receptors of the oncomiracidium and elicits a neuromuscular response, which, as observed by Macdonald (1974), leads to sudden extension of the body of the larva and dislodgement of the operculum. It has been shown in the present study that several analogs of urea, namely, MU [molecular weight (MW) = 74.08], DU (MW = 88.11), TMU (MW = 116.16), TU (MW = 76.12), TMTU (MW = 132.23), and PTU (MW = 152.22), all at concentrations of 1 mM in PSW, do not stimulate hatching in fully developed eggs rang-

TABLE 1. EFFECT OF UREA ANALOGS ON EGG HATCHING AND ACTIVITY OF UNHATCHED LARVAE OF *Acanthocotyle lobianchi*[a]

| Expt | Initial treatment (1 mM solutions of urea analogs) | Age of eggs (days) | No. of eggs tested | No hatched in initial treatment | Movements within egg | Hatch in initial treatment % | Hatch in posttreatment No. | Hatch in posttreatment % |
|---|---|---|---|---|---|---|---|---|
| 1 | MU | 27 | 12 | 0 | + | 0 | 10 | 83.3 |
| 2 | MU | 46 | 10 | 0 | + | 0 | 7 | 70 |
| 3 | MU | 75 | 8 | 0 | + | 0 | 8 | 100 |
| 4 | DU | 27 | 14 | 0 | + | 0 | 10 | 71.4 |
| 5 | DU | 46 | 10 | 0 | + | 0 | 10 | 100 |
| 6 | DU | 75 | 8 | 0 | + | 0 | 8 | 100 |
| 7 | TMU | 27 | 6 | 0 | + | 0 | 3 | 50 |
| 8 | TMU | 46 | 5 | 0 | + | 0 | 3 | 60 |
| 9 | TMU | 75 | 6 | 0 | + | 0 | 6 | 100 |
| 10 | TU | 27 | 20 | 0 | + | 0 | 14 | 70 |
| 11 | TU | 46 | 7 | 0 | + | 0 | 6 | 85.7 |
| 12 | TU | 75 | 6 | 0 | + | 0 | 6 | 100 |
| 13 | TMTU | 27 | 5 | 0 | + | 0 | 5 | 100 |
| 14 | TMTU | 46 | 6 | 0 | + | 0 | 5 | 83.3 |
| 15 | TMTU | 75 | 7 | 0 | + | 0 | 5 | 71.4 |
| 16 | PTU | 27 | 17 | 0 | − | 0 | 14 | 82.4 |
| 17 | PTU | 46 | 9 | 0 | − | 0 | 8 | 88.9 |
| 18 | PTU | 75 | 7 | 0 | − | 0 | 3 | 42.9 |
| | Control (PSW) | 27 | 11 | 0 | − | 0 | 9 | 81.8 |
| | Control (PSW) | 46 | 8 | 0 | − | 0 | 8 | 100 |
| | Control (PSW) | 75 | 7 | 0 | − | 0 | 7 | 100 |

[a] Only one set of controls for eggs of each age group is shown. +, movements of larvae observed within some eggs; −, no movements of larvae observed within any eggs.

ing in age from 27 to 75 days. That this is not the consequence of an irreversible effect on the ability of the larvae to hatch was demonstrated by the observation that between 42.9 and 100% of the posttreated eggs hatched in the presence of a 1 mM solution of Analar urea in PSW.

The molecular weights of all the analogs of urea used in these experiments were greater than that of urea (Figure 1), and it is possible that some or all of these substances diffuse too slowly into the egg to stimulate the larva or completely fail to penetrate. However, some degree of penetration by most of these chemicals seems likely because, with the exception of PTU, which has the largest molecular weight of the chemicals tested, all the analogs stimulated movements of larvae within the eggs. If it is assumed that the analogs are able to enter the intact egg, then some inferences can be drawn about the nature of the chemoreceptor responsible for the initiation of the hatching behavior. The lack of a hatching response to TU and PTU suggests that the amino group in the urea molecule is not alone responsible for activating the chemoreceptor and, similarly, the failure to respond to DU and TMU suggests it is unlikely that the active part of the urea molecule is the carboxyl group. Molecules such as MU with both an amino group and a carboxyl group also appear to be ineffective as hatching stimulants, and Kearn and Macdonald (1976) showed that substances likely to be excreted by elasmobranchs, such as trimethylamine oxide, ammonium chloride, and some amino-acids, fail to stimulate hatching. Thus, the oncomiracidial chemoreceptor responsible for initiating the hatching process appears to have a high specificity for the urea molecule, failing to respond to molecules similar to urea with amino groups only, with carboxyl groups only, or with both of these groups.

It is interesting that all of the analogs of urea except PTU appear to stimulate movement of the larva but fail to trigger the extension of the body of the larva that dislodges the operculum. It is possible that urea-mediated hatching is a two-stage process involving, first, activation of the larva and, second, extension of the body. Perhaps the urea analogs (with the exception of PTU) initiate the first stage but fail to stimulate extension of the body of the larva.

Whittington (1987) determined that eggs of the microbothriid *Leptocotyle minor* and of the hexabothriid *Hexabothrium appendiculatum* from the skin and gills, respectively, of the same elasmobranch host, *Scyliorhinus canicula*, also hatch rapidly (within 10–50 sec) after application of urea. However, eggs of *L. minor* also hatch after treatment with a 100 mM solution of TU in PSW but not with a 5 mM solution of TU in PSW. He suggested that the observed hatching response of *L. minor* eggs to relatively high concentrations of TU might indicate a disposition for thiourea to bind to receptor sites which usually bind urea, or that the eggs might have responded to an impurity, possibly urea, in the commercial sample of thiourea. In the present study, eggs of *A. lobianchi* were not tested with concentrations of urea analogs other than 1 mM, and it remains to

be determined whether higher concentrations of these chemicals stimulate hatching.

Kearn and Macdonald (1976) found that the susceptibility of eggs of *A. lobianchi* to stimulation with urea appears to increase with the age of the egg. A similar trend was detected in the present study. However, despite the enhanced capacity of the 75-day-old eggs to respond to urea, none reacted to any of the analogs tested. Curiously, not all 75-day-old eggs treated initially with TMTU and PTU hatched on posttreatment with urea. This may indicate some toxicity of TMTU and PTU for older eggs, but toxicity does not seem to be a feature of TU treatment, at least at a concentration of 1 mM. This contrasts with the finding of Simpson and Ogden (1932) that TU is toxic to the elasmobranch heart.

*Acknowledgments*—We thank the director and staff of the Laboratory of the Marine Biological Association of the United Kingdom at Plymouth for their hospitality and facilities and especially Mr. J.E. Green who helped to collect the parasites. We are indebted to Professor C. Arme, Department of Biological Sciences, University of Keele, U.K., who suggested that we make this investigation, and we acknowledge the advice of Professor C. Dobson, Department of Parasitology, University of Queensland, and Dr. H. Fazldeen, Department of Chemistry, University of Queensland. We are grateful to Dr. P.C. Croghan, University of East Anglia, for comments on the manuscript and to Dr. R.D. Adlard, Department of Parasitology, University of Queensland, for help in preparing Figure 1. This study was conducted during the tenure of a Science and Engineering Research Council Studentship by I.D.W. at the University of East Anglia.

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