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Progress towards characterization of a predator/prey kairomone: *Daphnia pulex* and *Chaoborus americanus*

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Abstract

Predatory larvae of the midge *Chaoborus americanus* induce an antipredator defense ('neckteeth') in prey individuals of the cladoceran *Daphnia pulex*. The signal for presence of predator is a water-soluble chemical. We provide evidence that this kairomone originates in the intestinal tract of the predator. The active compound is an organic molecule of intermediate polarity which is heat stable and partially destroyed by acid and base digestion. It is stable to digestion by the general peptidase Pronase. Hydroxyl groups, but not primary amines, carbonyls or thiols are essential to activity. Low-pressure liquid chromatography on a reverse-phase silica gel (Amicon Matrex C-18) column suggests there may be more than one active component.

Introduction

Induced antipredator morphologies in aquatic organisms were first recognized in rotifers (Gilbert, 1966). Since then many such defenses have been found, in a wide range of prey (e.g. Grant & Bayley, 1981; Krueger & Dodson, 1981; Harvell, 1984; Hebert & Grewe, 1985; Washburne *et al.*, 1988; for a review, see Havel, 1987). It is the evolutionary achievement of a phenotypic, often developmental, plasticity which makes possible the antipredator morphology. Such indirect predator effects might be expected to have a significant impact on community interactions (Kerfoot & Sih, 1987; Crowder *et al.*, 1988). Many, if not all such adaptive responses are mediated, in freshwater systems, by a semiochemical cue ('kairomone') which signals the presence of predator. In no cases have the kairomone been identified.

Daphnia pulex (Leydig), in the presence of predatory larvae of the midge *Chaoborus americanus* (Johannsen), develop a carapace pedestal with spines called 'neckteeth' (Krueger & Dodson, 1981). Havel & Dodson (1984 and 1987) demonstrated the adaptive significance of carrying neckteeth (increased likelihood of escape) and noted possible reproductive costs associated with the defensive morphology.

Both Krueger & Dodson (1981) and Hebert & Grewe (1985) have shown that the cue used by *Daphnia* for the presence of *Chaoborus* is a water-soluble chemical. Identification of the kairomone will allow correlation of *in situ* kairomone concentration with degree of expression of the antipredator defense. Use of a defined chemical treat-

ment will also grant greater precision in microcosm and mesocosm manipulations as well as autecological studies such as life table analyses, tests of behavioral responses or interclonal comparisons. The phenotypic response patterns of different clones or species to authentic kairomone will provide a chemical perspective on *Daphnia* evolution and coevolution. Elucidation of the cellular and molecular mechanisms of the morphological response using e.g. radiolabelled or photoaffinity labelled kairomone also awaits its identification. The purpose of this paper is to communicate what we have so far learned about the nature of this signal in the system *Daphnia pulex* clone SBL/*Chaoborus americanus*.

Methods

Bioassay

Daphnia pulex clone SBL, isolated from a single female collected from α-Gardner pond, Univ. of Wisconsin Arboretum, Dane Co., Wi., was used in our bioassays to test for necktooth-inducing activity. The purity of the study animals was regularly verified by means of cellulose acetate electrophoretic phenotypes of six enzymes as per Hebert, Ward and Wieder (1988). SBL clone scored as follows: *MPI*, 33; *AO*, 55; *LDH*, 15; *FUM*, 55; *GOT*, 11; *PGM*, 33; *PGI*, 15. *Daphnia* were raised in laboratory cultures in 20-liter battery jars fed a mixture of predominately *Scenedesmus*, *Clorella*, *Ankistrodesmus* and *Selenastrum* from green water tanks containing guppies. Adults were screened from the culture and stage 1 eggs (Threlkeld, 1979) were removed from gravid females for an *in vitro* bioassay with culture techniques adapted from Obreshkove & Fraser (1940).

Chaoborus americanus third and fourth instar larvae were collected in Tender Bog Lake of in the Univ. of Notre Dame Environmental Research Center in Gogebic County, northern Michigan. *Chaoborus* were held in a cold room (4 °C) for up to several months before bringing them up to room temperature, feeding them liberally with *Daphnia* for 48 hours, and sacrificing them. Extract of the *Chaoborus* was made as per Herbert & Grewe (1985), which yields (per gram of *Chaoborus*) 2 ml of a clear, slightly yellow filtrate containing solutes of < 500 MW. Extract of other organisms was made in an identical way. Extracts were frozen (minus 10 °C) for assay or purification and were stable at that temperature for six months or more.

We lyophilized a 1200 ml aliquot of about 15 liters of distilled water in which approximately 8–10000 *Chaoborus* were kept, well fed, at room temperature for 48 hours. The resulting brownish powder was dissolved in 20 ml distilled water and treated to the same series of filtrations as *Chaoborus* extract.

Bioassay for the presence of active kairomone was done by applying extract to a single clutch of eggs (4–25, usually 6–12) in a small petri dish containing 10 ml of filtered (Whatman 1) and boiled *Daphnia* culture water. The eggs were placed in an incubator (21 °C, 12 hour light/dark) for approximately 72 hours with one additional treatment on a daily basis. After 3 days the second instar neonates were scored for necktooth induction. Survivorship to second instar juveniles averages 75–80%; any replicates with less than 50% survival were not included in analyses. Five replicates (clutches) were used per treatment. Preliminary studies indicated that at very low concentrations of extract, either a small bulge or a full pedestal without spines formed in the necktooth region. Increased sensitivity was attained by adopting a scoring procedure which also scored these bulges or pedestals without actual spines. This scoring procedure was as follows: Score 0 for no effect; score 1 for slight bulge in the necktooth area; score 2 for full-sized pedestal without spines; score 4 for one or more spines on a pedestal. The number of individuals at each score is then multiplied times that score, this sum is divided by four and again divided by the total number of individuals in that replicate to yield percent induction. By this system a replicate in which all neonates showed only slight bulges in the necktooth area would score as 25% induction; all showing pedestals 50% induction and all

showing neckteeth 100% induction. Positive (*Chaoborus* extract) and negative (distilled water) controls were included in all bioassays.

Chaoborus dissection

In order to more precisely determine the source of the active compound(s) produced by the predator, third and fourth instar larvae of *Chaoborus americanus* were fed excess *Daphnia pulex* for 48 hours. One hundred and twenty-five larvae were then dissected under a dissecting microscope as follows. The head and thorax (portion 1) were first removed, then the anal segment (portion 3). With forceps the intestine and attached Malpighian tubules were then pulled forward through the abdominal segments, leaving the abdominal body wall (portion 2). The midgut (4) was cut from the hindgut (5) at the colon. The Malpighian tubules, attached to the midgut just anterior of the colon, were included with the hindgut, except for a small portion at their point of attachment, which went with the midgut. The foregut was left in the thorax. Figure 1 shows the location of portions 1–5.

All individuals dissected had visible midgut digestive contents; about half (46%) had clearly full foreguts. All portions were kept on ice until being blotted dry, weighed, and frozen in 2.5 ml of distilled water per portion. Extract of these portions was then made as previously described, and 50 microliter aliquots were used in our standard bioassay procedure.

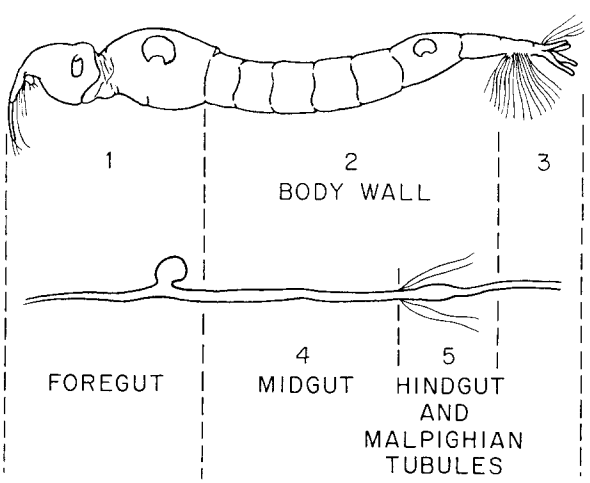

Fig. 1. Plan of *Chaoborus* dissection.

Morphogen characterization

Chemical and temperature stability experiments and solubility studies of *Chaoborus* extract were replicated at least twice; data shown are for means of replications. Treatments included both positive controls (extract treated with all but the critical reagent) and negative controls (all reagents included except extract). In no case did negative controls show significant induction.

Extract was determined to be about 2 mg/ml solids. This filtrate has passed through a 500 MW cut-off membrane; for an average MW of 250, 2 mg/ml represents an 8 mMolar solution. These values were used in the functional group analyses below to assure that excess reagents were included in the reaction vessels.

Calbiochem *Streptomyces griseus* Pronase (which splits peptide bonds) was dissolved at 0.1 mg/ml in pH 8.0 1% ammonium bicarbonate buffer and incubated with an equal volume of extract for 8 hours at 40 °C. Pronase which had been boiled for 10 minutes was used as a positive control.

To test the effect of acetylating hydroxyl groups, base-catalyzed acetylation was done as per Kay *et al.* (1983). Three mls of extract were evaporated to dryness under vacuum and taken up into 3 mls of pyridine. Two each 0.5 ml replicates were withdrawn as positive controls, 2 each 0.9 ml replicates as treatments. To each treatment was added 1 ml acetic anhydride and acetylation was allowed to proceed for one hour at 60 °C. Positive controls were incubated at 60 °C for one hour without acetic anhydride; negative controls were 0.9 ml distilled water instead of extract, otherwise treated exactly as treatments. After one hour all replicates received 1 ml each distilled water to stop the acetylation reaction. Replicates were evaporated under vacuum and taken up into starting volumes of distilled water (0.5 to 0.9 ml) for bioassay.

Fluorescamine (Sigma F-9015), which under the conditions used herein irreversibly reacts with primary or secondary amines or thiol groups, was reacted with extract with slight modification of the procedure of Gottschalk & Sonneborn (1985). One ml of extract evaporated under vacuum per replicate was taken up into 1 ml pH 9.0 buffer (.025 M $NaHCO_3$/NaOH), to which was added 30 microliters of 10 mg/100 microliters fluorescamine in acetone, dissolved just before use. This was reacted at room temperature (21 °C) for 15 min., the pH adjusted to about 5.0 (the approximate pH of native extract) with 0.1 M HCl, followed by evaporation under vacuum and redissolving the products in 1 ml distilled water for bioassay. Negative controls were buffer without extract and positive controls received 25 microliter acetone without fluorescamine.

Sodium borohydride reduces carbonyl groups to alcohols. Borohydride reduction was performed based on procedures in Fieser & Fieser (1967). 0.5 ml of extract per replicate was evaporated under vacuum and taken up into 2 ml absolute methanol, either containing 0.1 M $NaBH_4$ (treatment) or no $NaBH_4$ (positive controls) and reacted at room temperature for 30 min. The reaction was stopped by adding 40 microliters glacial acetic acid per replicate; after vacuum evaporation, the products were taken up into 0.5 ml distilled water for bioassay.

Purification of the active component(s) of *Chaoborus* extract involved a large-scale preparative LPLC (low pressure liquid chromatography) column consisting of 200 grams of Amicon Matrex Silica C18 50 micron, 60 Angstrom pore size reverse-phase silica gel. Column size was 4.5 cm diameter × 28 cm height. We started with an LPLC column of only 10 grams of the same solid support (1.4 cm dia. × 12 cm height); this column was used to analyze lyophilized *Chaoborus* culture water. Amount of material applied and volume of eluents were scaled accordingly. The column was equilibrated at 25% methanol in nanopure H_2O. For the 200 gram column, thirty mls of *Chaoborus* extract with 10 ml methanol (Burdick and Jackson High Purity) were applied to the column at room temperature. Five hundred ml of 25% methanol was applied to the column under about 4 psi dry nitrogen, with a flow rate of 4 ml min. Fractions were collected at 100 ml intervals. Five hundred ml of 50% methanol was then applied to the column under the same conditions, followed by 500 ml of absolute methanol, which gave a 10 ml/min flow rate at the same pressure. With a reverse-phase system, compounds eluting with the 25% methanol wash would be the more polar fractions; those with the 50% wash of medium polarity and those coming off with absolute methanol the least polar. Fractions were individually vacuum evaporated, taken up in 5 ml nanopure water and frozen for bioassay or further purification. Optical densities were measured on an Hitachi 100-60 UV/vis spectrophotometer, with proper dilutions made to bring measured absorbances below 0.500.

Results

Individual *Daphnia* respond to the presence of *Chaoborus* larvae by some chemical factor released by the predator (Krueger & Dodson, 1981). Presumably this factor is some metabolite excreted by the predator. Table 1 gives some information about the source of this metabolite. On an activity per mg tissue basis, the midgut is seen as the most active portion of the predator. Hindgut and Malpighian tubules, dissected together, have approximately half the midgut activity, on a wet mass basis; the high variance in

Table 1. Bioactivity of various portions of Chaoborus larvae

Treatment	Total tissue mass (mg in 2.5 ml)	% Induction ± s.e. 50 µl treatment	% Induction/ mg tissue in 50 µl
Control	0	0 ± 0	–
Portion 1	317	87 ± 9	13.7
2	330	51 ± 14	7.7
3	119	0 ± 0	0
4	116	89 ± 5	38.4
5	105	53 ± 28	19.6

the bioassay of portion 5 is unexplained. Previous dissections (data not shown) indicated that the head itself had no activity; some small activity is therefore found in the thorax. This may or may not be due to contamination, during dissection, from the midgut. Likewise the abdominal body section, less the gut, has very low activity, which may be contaminant from the gut. The anal segment is without activity.

All necktooth-inducing activity passed through the Amicon membrane filter, indicating no active molecules larger than 500 MW.

Figure 2 shows the dose-response curve giving percent induction vs. volume of *Chaoborus* extract applied per daily treatment. Response appears linear at lower levels of treatment before levelling off at nearly 100% induction. A significant response is detectable at 0.5 microliter treatment, which in 10 ml of culture medium represents a 5×10^4-fold dilution of extract.

Table 2 shows the results of temperature and acid/base treatment of extract and bioassays of extracts made from *Aedes vezans*, *Daphnia pulex*, and *Chaoborus* pupae. Ashing to constant weight completely destroys activity; this suggests that the active fraction is an organic rather than inorganic molecule. That this is a relatively stable compound(s) is shown by its stability for long periods at -10 °C and during boiling. Significant activity is also retained after boiling at pH2 and 12 for two hours, eliminating esters as the active form of the compound. The inability to partition active components into diethyl ether or chloroform suggests a rather polar, lipid-insoluble compound or compounds.

Table 2. Results of various treatments of *Chaoborus* extract and bioassay of other extracts.

Treatment	% of untreated activity
Ashing	0 %
Boiled dry, reconsituted	101 %
Boiled 4 hrs, pH 7	86 %
Boiled 2 hrs, pH 2	40 %
Boiled 2 hrs, pH 12	30 %
Diethyl ether extraction (3 × volume)	
Ether fraction	10 %
Aqueous fraction	99 %
Chloroform extraction (2 × volume)	
Chloroform fraction	0 %
Aqueous fraction	130 %
Extract made from:	
Aedes vexans late instar larvae	0 %
Daphnia pulex	0 %
Chaoborus americanus pupae*	17 %

* one replicate only

5 microliters used in standard bioassay for all treatments except *Aedes*, *Daphnia* and *Chaoborus* extracts which were 50 microliters.

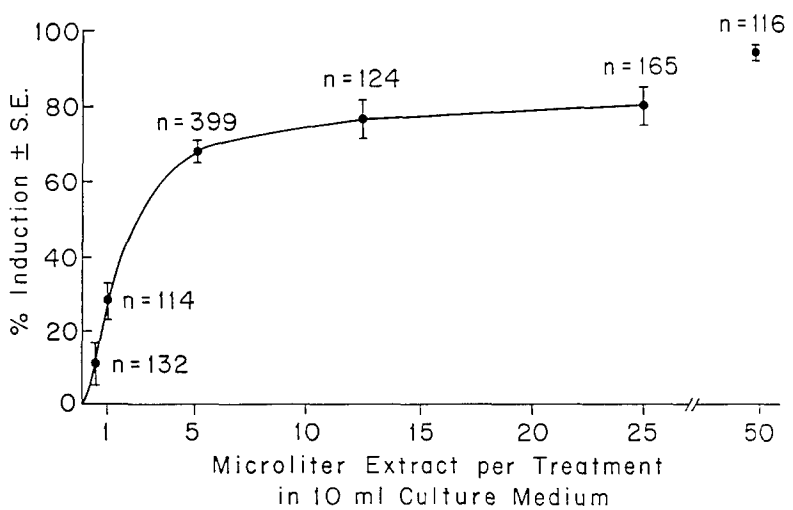

Fig. 2. Dose-response curve. Percent induction vs. microliters of *Chaoborus* extract applied per day to 10 ml culture medium.

Extract made from herbivorous larvae of *Aedes vexans* and from *Daphnia pulex* itself has no activity. Extract of *Chaoborus americanus* pupae has greatly reduced activity.

Table 3 shows that treatment of extract with pronase does not decrease activity; the active compound is not likely therefore to be either a small peptide or ester of an amino acid or peptide (pronase has been shown to have some esterase activity – see Narahashi, 1970). Base-catalyzed acetylation, which acetylates hydroxyl, primary or secondary amines or thiols (Knapp, 1979) destroys extract activity.

Fluorescamine has been shown to react irreversibly with primary and secondary amines and thiols, and reversibly with alcohols (Castell *et al.*, 1979). The insensitivity of inducing activity to fluorescamine suggests neither primary nor secondary amines nor thiol groups are essential to activity. In aqueous solution, fluroescamine itself undergoes rather rapid hydrolysis, which competes with the more rapid irreversible reaction with amines and thiols (Castell *et al.*, 1979). It is this hydrolysis which necessitates the use of excess fluorescamine in the reaction. But because of this competing hydrolysis, the reversible reaction with any alcohols present would not be expected to lead to a stable product. Taken together the acetylation and fluorescamine results indicate the presence of one or more hydroxyl groups on the active compound(s) which are necessary for biological activity.

Table 3. Results of Functional Group Analyses

Reaction	Reacts With	% of Untreated Activity
Pronase digestion	peptides	116%
Base-catalyzed acetylation	OH, SH or NH_2	1%
Fluorescamine	SH or NH_2	100%
Sodium Borohydride reduction	CO	111%

For reaction conditions, see Methods; 20 microliters used in standard bioassay procedure for acetylation reaction, all others 5 microliters.

Sodium borohydride, under the reaction conditions used, reduces only aldehydes and ketones (Fieser & Fieser, 1967) and does not affect biological activity. Either there are no carbonyls on the active compound(s) or their presence is not required for biological activity.

The results of applying extract to the LPLC column are shown in Fig. 3. About 94% of the measurable absorbance at 225 nm is eluted from the column at 25% methanol. Activity adheres to the column with the 25% eluent but comes off in the 50% methanol wash. This is consistent with a compound or compounds of intermediate polarity. It is not possible to test the absolute methanol wash for activity, as these fractions contain some component (believed to originate in the Amicon Matrex solid support) which is toxic to the developing embryos. However, recovery calculations suggest that 90% or more of activity applied to the column is routinely recovered in the 50% methanol wash. The presence of two peaks of activity, as shown in Fig. 2, suggests more than one active compound, with the earlier peak corresponding to a more polar component, the latter a less polar component. This pattern of two peaks of activity is replicatable; other explanations than two active species, such as the comigration of an inhibitor in fraction 7, are also possible.

LPLC of the lypophilized *Chaoborus* water on a smaller column resulted in activity ($17 \pm 3\%$ induction) only in fraction 7 (10 ml fractions, data not shown). That only one peak of activity was found in the lyophilized *Chaoborus* water may be due either to absence of the second activity in the water or its presence at a level too low to detect.

Discussion

The distribution of inducing activity within dissected *Chaoborus* shown in Table 1 is consistent with its origin within the digestive tract of the predator, either as a component of digestion or some metabolite excreted into the midgut. The evidence does not support the compound as a

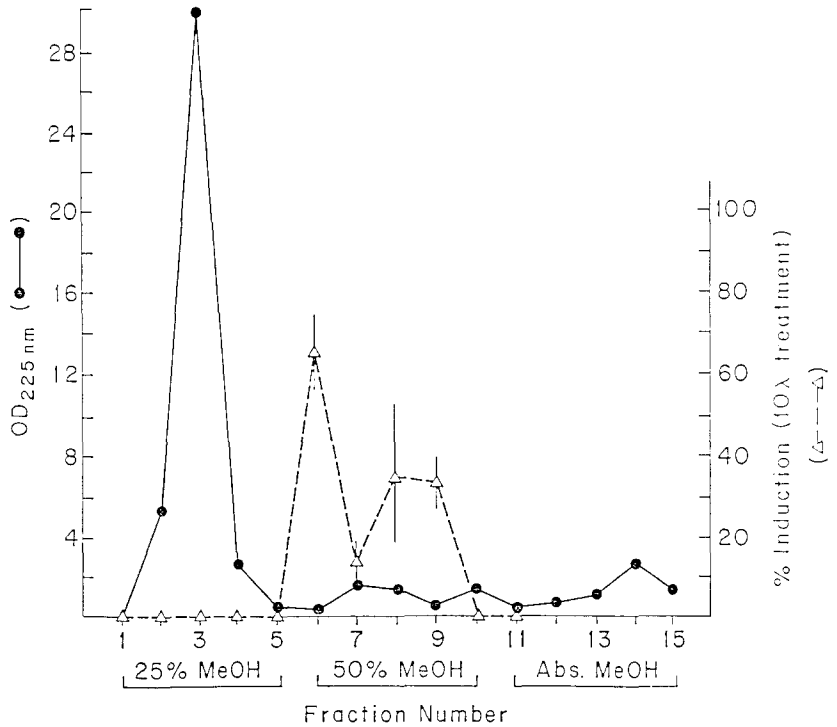

Fig. 3. LPLC of 30 mls *Chaoborus* extract on 200 grams Amicon Matrex Silica C18. Five each 100 ml fractions collected at 25% methanol, 50% methanol and absolute methanol. Flow rate 4 ml/min (10 ml/min at absolute methanol).

metabolite excreted via the Malphigean tubules. Table 2 indicates that it is not the products of digestion of *Daphnia* itself which are the source of activity. Although in this experiment nearly twice the midguts had visible digestive contents as the foreguts, in previous experiments in which the foregut and midgut were equally full, the same ratio of activities was found. Kaiser (1984) notes that the digestive juices in *Chaoborus* are produced in the abdominal region approximately four body segments posterior to the thorax and via reverse-peristalsis are moved forward into the foregut during digestion. Such a process is consistent with the speculation that the active morphogen is some component of the predator digestive juices.

Figure 3 suggests at least two active forms of kairomone; a more polar form eluting earlier in LPLC while the second, less polar, elutes later. Whether both forms are naturally released into the water or are only extracted when the larvae are boiled as in our procedure remains to be seen. Our experience with the less polar component(s) indicates they are somewhat less stable than the more polar component(s). It may also be that treatment at extreme pHs (see Table 2) differentially destroys one form or another. It will be of some interest to see whether the same compound or compounds are active in different clones of *Daphnia pulex* and in different *Daphnia* species which show this or similar induced responses.

Many hormone receptors and enzymes show dose-response curves very similar to Fig. 2 and usually have their half-maximal response near or somewhat above physiological concentration of hormone or substrate (Martin, 1987; Stryer, 1981). In this region they are most sensitive to changes in hormone or substrate concentration. By analogy we would expect the response shown by *Daphnia* to the presence of *Chaoborus* to show about 50% induction at some natural level of *Chaoborus* density. Havel (1985) sampled a pond ten times from April 5 to July 19 and reports an average *Chaoborus* density of 2.2 $\pm$ 0.7 per liter.

He records 50% necktooth induction for *Daphnia pulex* in that pond at about 1.5 to 2.0 predators per liter. However, we might expect such responses to vary from one clone to another.

Production of the morphological defense has a cost; allocation of resources to it may be at the expense of reproductive rate (Havel & Dodson, 1987; Kerfoot, 1977). Under conditions of both predation and competition for limited resources we would therefore expect an inducible defense to be most advantageous. The cue relied on for the presence of predator would therefore likely be specific to the predator. It is clear from Table 1 that it is not just the act of predation, by releasing cellular fluids from *Daphnia*, which produces the kairomone. We (unpubl. data) have evidence that extracts of *Chaoborus* fed any of a variety of alternative prey show equivalent inducing activity. Walls and Ketola (1989) fed *Chaoborus crystallinus* a *Eudioptamus* sp. and found equivalent activity to *Chaoborus* which had been fed *Daphnia*. It will be of some interest, once the kairomone is identified, to locate its exact source in the predator.

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