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ACETAMINOPHEN-INDUCED DEPLETION OF GLUTATHIONE AND CYSTEINE IN THE AGING MOUSE KIDNEY*

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Abstract—Glutathione (GSH) plays an essential role in the detoxification of acetaminophen (APAP) and the prevention of APAP-induced toxicity in the kidney. Our previous results demonstrated that a GSH deficiency is a general property of aging tissues, including the kidney, suggesting a hypothesis that senescent organisms are at greater risk to APAP-induced renal damage. To test this, C57BL/6NIA mice of different ages through the life span were injected with various doses of APAP, and the extent of GSH and cysteine (Cys) depletion and recovery were determined. At time intervals up to 24 hr, kidney cortex samples were obtained, processed and analyzed for glutathione status, namely GSH, glutathione disulfide (GSSG), Cys and cystine, using an HPLC method with dual electrochemical detection. In the uninjected controls, GSH and Cys concentrations decreased about 30% in the aging mouse, but the GSSG and cystine levels were unchanged during the life span. APAP administration depleted the kidney GSH and Cys contents in a dose- and time-dependent manner. Four hours after APAP administration, GSH levels of the young, growing (3- to 6-month) and the mature (12-month) mice decreased 34 and 58%, respectively, and recovered to near control values by 24 hr (95 and 98%). In contrast, the extent of depletion in old (31-month) mice was greater (64%) and the 24-hr recovery was less, returning only to 56%. Likewise, Cys levels of the young and mature mice decreased 49 and 65%, respectively, 4 hr following APAP, and increased to 99 and 85% by 24 hr. In contrast, in old mice, there was a 78% depletion after 4 hr followed by a recovery of only 65% by 24 hr. These results demonstrated clearly that in the aging mouse kidney, a GSH and Cys deficiency occurs that is accompanied by an impaired APAP detoxification capacity.

Acetaminophen (APAP‖) is a widely used antipyretic and analgesic that in high doses can result in extensive kidney and liver injury [1, 2]. In both organs, APAP-induced tissue necrosis is believed to result from the covalent binding of a reactive quinonimine metabolite of APAP to cellular macromolecules. The metabolism of APAP to this reactive intermediate involves a cytochrome P450-dependent process which may be preceded by deacetylation of APAP to *p*-aminophenol in the kidney of some species [3–5].

Glutathione (GSH) plays an essential role in the detoxification of these reactive APAP-intermediates in both the liver and the kidney [6, 7]. The binding of GSH to activated APAP is an obligatory step in the detoxification process, and toxicity is not apparent until GSH stores are depleted [3, 8]. Accordingly, depletion of liver and kidney GSH prior to APAP administration potentiates its toxicity [3, 9].

Our previous findings demonstrated that a GSH deficiency is a general feature of many aging tissues including the liver and kidney [10–13]. Since GSH serves as the major detoxifying agent for the reactive metabolite of APAP in these tissues, it is likely that the detoxification capacity for APAP is impaired in old organisms. Indeed, clinical studies of elderly subjects have demonstrated that APAP elimination is diminished [14]. However, to our knowledge, there have been few studies which have examined the effect of aging on susceptibility to APAP toxicity in either humans or laboratory animals.

The objective of this study was to determine the effect of acute APAP administration on GSH status in the C57BL/6 mouse at different ages throughout the life span. Earlier, we reported that, in the liver, the ability to recover GSH stores after depletion by APAP was greatly impaired in old as opposed to mature mice [13]. These results demonstrated an aging-specific loss of APAP detoxification capacity in the liver that was correlated with a GSH deficiency state.

In the present paper, we report the GSH and cysteine (Cys) results obtained in the kidney. This organ was studied because it is a key target organ for APAP toxicity and also is the major site for the metabolism of GSH–APAP conjugates to secondary sulfur-containing metabolites such as Cys and *N*-acetylcysteine conjugates of APAP [15, 16].

MATERIALS AND METHODS

Animals. Animals on treatment protocols were described previously [13]. Male C57BL/6NIA mice,

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‖ Abbreviations: APAP, acetaminophen; GSH, glutathione; GSSG, glutathione disulfide; and Cys, cysteine.

a standard, well-characterized aging model, were obtained from the National Institute on Aging colony at Charles River Breeding Laboratories. The ages were based on their median survival time of 29–30 months and represented the growth (3 and 6 months), mature (12 and 26 months), and aging (31 months) periods of the life span. Every shipment and daily experimental design included mice from different age groups. All mice were clinically healthy, active upon receipt, and acclimated for a week in our animal care facility prior to experimentation. Up until the morning of the experiment, mice were fed a standard mouse-chow diet (Purina Chow) containing a nutritionally adequate, sulfur amino acid content and given water *ad lib*.

APAP administration and tissue preparation. Various doses of APAP in ethanol and propylene glycol (1:4) were injected i.p. into four animals of each age group. Injections were made routinely between 8:00 and 9:00 a.m. The mice were killed 4, 8, and 24 hr following administration of APAP by cervical dislocation, and their kidneys (both right and left) were excised quickly, decapsulated, rinsed in ice-cold 0.85% (w/v) saline, blotted dry, dissected to remove all non-cortex tissue, and weighed. The samples were kept cold (0–4°) and processed as quickly as possible to minimize auto-oxidation and degradation of GSH. Kidney homogenates (10%, w/v) were prepared in 5% (w/v) metaphosphoric acid and centrifuged to obtain acid-soluble fractions for the measurement of GSH, glutathione disulfide (GSSG), Cys and cystine. Recovery of known amounts of each analyte added to samples prior to homogenization was greater than 96% by this method.

Analysis of glutathione and cyst(e)ine. GSH, GSSG, Cys and cystine were quantified simultaneously by our HPLC method using a dual electrochemical detector of two Au/Hg electrodes in series [17]. In brief, 40-μL samples were applied onto a 25 cm × 4.6 mm, 5 μm ODS column which was eluted isocratically using a mobile phase of 96% (v/v) 0.1 M monochloroacetic acid (pH 3.0), 4% (v/v) methanol, and an ion-pairing reagent of 2.0 mM heptane sulfonic acid with a flow rate of 1 ml/min. The resultant profiles were compared in each daily analysis with profiles obtained with authentic standards.

Stability of GSH and Cys in kidney samples. The extent of GSH conversion to Cys in the kidney during sample processing was determined. Three 12-month-old mice were killed and their kidneys were removed as quickly as possible. After rinsing in ice-cold saline, each kidney was divided in half. One of the four halves from each mouse was processed immediately (3 min after cervical dislocation). The remaining three halves were incubated in saline at either 0, 21 or 37° and after 15, 30 and 60 min were chilled to 0°, homogenized and analyzed as above.

Chemicals. APAP, GSH, GSSG, Cys and cystine were obtained from the Sigma Chemical Co. (St. Louis, MO).

Data analysis. Glutathione, GSSG and cyst(e)ine concentrations were expressed on the basis of kidney weight. Standard methods of statistical analysis were applied, including correlation coefficients, coefficients of variation and Student's *t*-test [18]. Differences were considered significant if $P < 0.05$ (one-tail *t*-test).

RESULTS

Body and kidney weights. Body and kidney weights during the mouse life span are summarized in Table 1. As expected for growing animals, a 20–30% increase in these weights was observed from 3 to 12 months of age, and a slight 10% decrease in body weight was observed thereafter, a common phenomenon in aging rodents. There was a 15% growth increase in kidney weight from 3 to 6 months of age and no change from maturity through senescence. Kidney weight to body weight ratio was similar during the periods of growth and maturity. The slight increase in ratio with aging resulted from the decrease in body weight.

Stability of GSH and Cys in kidney samples. When kidneys were excised, placed immediately in ice-cold saline, and incubated up to 60 min, no changes were observed in the levels of GSH and Cys from the values obtained on samples processed immediately after removing the kidney (3 min after cervical dislocation of the mouse) (Fig. 1). When kidneys were incubated at 21° for 60 min, GSH levels decreased 43% while Cys levels increased 9% from initial values. At 37°, GSH levels decreased to 77% while Cys increased 36% from initial values. These results confirm that the standard conditions used in this study for sampling and processing kidney cortex samples are appropriate for the accurate determination of GSH and Cys levels. However, at higher temperatures, postmortem conversion of GSH to Cys occurred.

GSH, GSSG, Cys and cystine concentrations. Kidney GSH and Cys contents ranged from 1.59 to 2.66, and 0.747 to 1.27 μmol/g, respectively, as shown in Fig. 2. The general profile throughout the life span was similar for both GSH and Cys with a high level during growth decreasing to a maturation plateau and then declining further during senescence. The GSH and Cys concentrations decreased about 30% in the aged, 31-month-old, mouse and were significantly different from that of the mature mouse ($P < 0.05$).

Cystine contents in the kidney ranged from 69 to 98 nmol/g, and GSSG ranged from 63 to 69 nmol/g. Neither of these disulfides changed during the life span.

Dose and time effects of acetaminophen on GSH content. The time courses of the effects of APAP on kidney GSH and Cys contents of 6-month-old mice are shown in Fig. 3. APAP depleted GSH and Cys in a time- and dose-dependent manner. Maximal depletion occurred 1–4 hr after administration, followed by a recovery toward the original value by 24 hr at lower doses (300 and 375 mg/kg). However, after the highest APAP dose (500 mg/kg), both GSH and Cys levels remained low and did not recover.

Based on this dose–response and time–course data, a dose of 375 mg/kg was selected for subsequent experiments. Mice administered APAP at this dose show no major signs of renal toxicity after 48 hr by histological examination and measurement of

Table 1. Kidney and body weights during the mouse life span*

Age (months)	No. of mice	Kidney (mg)	Body (g)	Kidney/Body ($\times 10^2$)
3	31	185 ± 8.30†	27.1 ± 0.402†	0.683 ± 0.0306
6	34	217 ± 5.38	31.5 ± 0.498†	0.689 ± 0.0171
12	26	232 ± 6.87	36.4 ± 1.13	0.637 ± 0.0189
26	34	246 ± 7.24	32.8 ± 0.658†	0.750 ± 0.0221†
31	26	245 ± 7.90	32.0 ± 0.680†	0.766 ± 0.0247†

* Values are means ± SEM.
† $P < 0.05$ vs 12-month values.

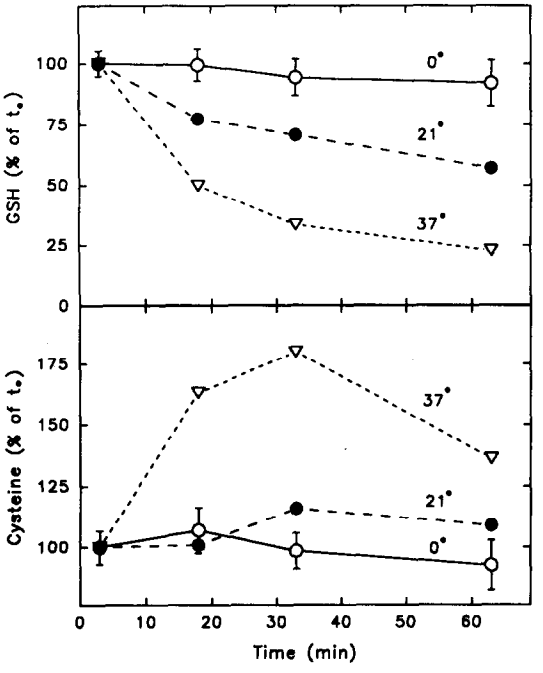

Fig. 1. Stability of glutathione and cysteine in excised mouse kidney. Glutathione and Cys concentrations were determined in samples of mouse kidney processed either immediately after the animal was killed (3 min) or after 15, 30 or 60 min of incubation in saline at 0, 21 or 37°. Values plotted are percent of pre-incubation levels (2.20 ± 0.110 μmol/g for GSH and 0.672 ± 0.0475 μmol/g for Cys). For the 0° time points, each point and bar represents the mean and SEM of 4 experiments. All other temperature and time points represent the results of a single experiment.

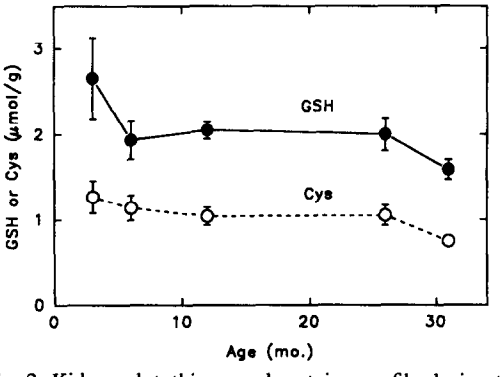

Fig. 2. Kidney glutathione and cysteine profile during the mouse life span. Glutathione and Cys concentrations were determined in samples from different ages through the life span. Each point and bar represent the mean ± SEM of 4–8 mice. Bars were omitted if the SEM was less than the size of the point.

covalent binding of APAP to renal macromolecules [5–7]. However, this dose has been shown to produce significant hepatic necrosis in 46% of mice and a mortality rate of 3% after 48 hr [7].

Aging differences in GSH and Cys levels after acetaminophen injection. Aging changes occurred in kidney GSH and Cys levels following APAP injection (Fig. 4). Maximal GSH depletion occurred at 4 hr after APAP administration and was age dependent. The percent depletion of GSH observed in the young mouse (34%) was significantly smaller than observed in either the mature (58%) or old mouse (64%) ($P < 0.001$). Kidney GSH levels of the 3- and 12-month age groups began to recover by 8 hr and rose to near control values by 24 hr (95 and 98%). In contrast, a lower 56% recovery occurred in the old (31-month) mouse, a value that was correlated with its low initial GSH content. Likewise, Cys levels of the young and mature mice decreased 49 and 65%, respectively, 4 hr following APAP, and rose to 99 and 85% by 24 hr. However, in old mice, there was a 78% depletion followed by recovery of only 65%.

Aging changes in the recovery of GSH and Cys levels. The percent GSH and Cys recoveries in kidney at 24 hr following APAP challenge are summarized in Fig. 5. The percent recovery values refer to the 24-hr GSH and Cys levels relative to those of the uninjected controls. The recovery of both GSH and Cys was highest during growth, decreased to a maturity plateau and further decreased during aging. Interestingly, these life-span patterns of kidney GSH and Cys recoveries coincide with their patterns of GSH and Cys concentrations.

DISCUSSION

The GSH deficiency of aging has been described

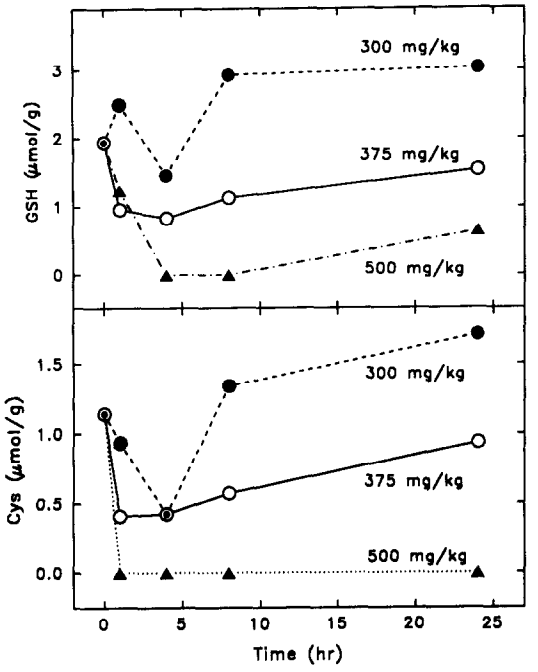

Fig. 3. Dose and time characteristics of acetaminophen depletion of glutathione and cysteine in kidney. Acetaminophen at 300, 375 and 500 mg/kg was administered i.p. to 6-month-old mice, and their GSH and Cys levels were measured 1, 4, 8 and 24 hr later. Each dose/time point represents a single animal.

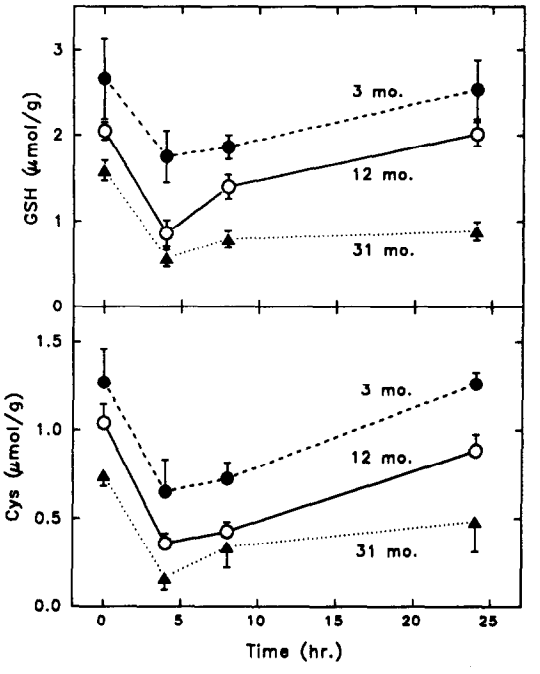

Fig. 4. Aging differences in kidney glutathione and cysteine levels after acetaminophen injection. Mice were given APAP (375 mg/kg) i.p., and their GSH and Cys levels were measured 4, 8 and 24 hr later. Each point and bar represent the mean ± SEM of 3–6 mice in 3-, 12-, and 31-month age groups. Bars were omitted if SEM was less than the size of the point.

in a variety of tissues from several aging animal models including the adult mosquito (*Aetes aegypti*) [11], adult housefly [19], mouse (C57BL/6) [10, 12, 13, 20, 21], and rat [22], and in lens tissue [23] and blood [24, 25] from elderly human subjects. The current results confirm our earlier findings of an aging-specific decrease in GSH in the mouse kidney. It is important to note that this deficiency is a post-maturational event that is not evident until old age. Further, these decreases probably reflect a biological aging phenomenon rather than a manifestation of a disease process, because there were no gross abnormal pathologies observed in any animal in this study.

The finding that APAP-induced depletion of kidney GSH is enhanced in old age supports our hypothesis that the GSH-related detoxification capacity for APAP in the kidney is impaired in aging organisms. Of particular interest was the loss of ability in old mice to recover from the APAP-induced decrease in GSH levels. This suggests that old mice remain in a vulnerable state with a minimal capacity for detoxification even 24 hr after APAP insult.

A similar loss of recoverability was also observed in the liver [13]. Four hours after APAP administration, hepatic GSH levels were depleted 70–80% in mice of all ages. After 24 hr, levels recovered 94% in young, 66% in mature, and only 41% in old animals. These aging-impairments in GSH recovery in liver and kidney are most likely caused by decreases in GSH biosynthetic capacity, as previously found in our aging mosquito model [26].

Although a number of studies have examined the renal metabolism and toxicity of APAP in growing

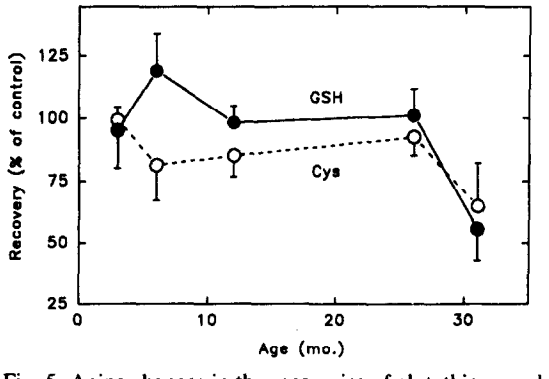

Fig. 5. Aging changes in the recoveries of glutathione and cysteine levels after acetaminophen injection. Mice were given APAP (375 mg/kg) i.p., and their kidney GSH and Cys levels were measured 24 hr later. Percent recovery was based on the 24-hr levels divided by age-matched uninjected control levels. Absolute values for uninjected control mice are provided in the legend of Fig. 1. Each point and bar represent the mean ± SEM of 3–6 mice. Bars were omitted if the SEM was less than the size of the point.

and mature animals, there have been only a few reports regarding senescent organisms. Consistent with our findings, Beierschmitt *et al.* [27, 28] demonstrated an enhanced susceptibility to APAP-induced renal toxicity in old (22–25-month) Fisher-344 rats compared to mature (12- to 15-month) animals. Although this increased toxicity could not be accounted for by changes in the metabolism of APAP, increases in the plasma levels of APAP and APAP glucuronide in old animals suggested that differences in APAP pharmacokinetics were involved.

In another study, the formation and elimination of APAP metabolites were examined in Fisher-344 rats of ages 5, 14 and 25 months [29]. When correlated with animal age, a decrease in sulfation and an increase in glucuronidation of APAP and no change in APAP mercapturate formation were reported. However, when the data are analyzed in a more appropriate manner for assessing aging changes, using the mature 14-month adult as the reference for comparison with the 25-month-old rats, a significant decrease in the excretion of APAP mercapturate of $> 20\%$ is observed. Although this decrease is consistent with our present results, the conclusions which can be drawn are limited as mecapturate formation is also dependent on the formation of APAP-glucuronide and APAP sulfate conjugates and on the oxidation of APAP by cytochrome P450 in both the liver and kidney.

Recently, hepatic and renal conjugation activities associated with APAP detoxification were examined in Sprague–Dawley rats of different ages throughout the life span [30]. The results demonstrated that APAP detoxification by conjugation with glucuronic acid or sulfate in both liver and kidney were unchanged during aging. Although liver glutathione *S*-transferase activity for 1-chloro-2,4-dinitrobenzene (CDNB) was also unchanged during aging, kidney levels were decreased $> 45\%$ in the old rats. Despite this decrease in the kidney, the authors concluded that the GSH pathway is not likely involved in the aging-enhancement of APAP-induced renal toxicity based on the assumption that the rate of formation of reactive APAP metabolites is slow.

However, detoxification by GSH conjugation is dependent not only on GSH *S*-transferase but also on the tissue concentration of GSH. Indeed, GSH levels and turnover are the critical factors regulating APAP toxicity as evidenced by the occurrence of an APAP-induced depletion of GSH prior to the observation of APAP toxicity and the protective effect of *N*-acetylcysteine and GSH against APAP-induced hepatotoxicity [31–33]. This evidence together with the present results suggest that a loss in GSH-related detoxification capacity is a cause of the aging-related increase in renal toxicity.

The aging impairment of GSH-related detoxification capacity in the kidney could have important implications in human subjects considering the frequent use of prescription and over-the-counter drugs and the increased incidence of adverse drug reactions observed in elderly populations [34]. Although APAP is normally considered a safe drug in humans, therapeutic doses have been reported to induce renal lesions [35–37]. Decreases in hepatic GSH have been observed after doses of 0.5 to 3.0 g in healthy humans [38] and acute hepatic injury and lethality have been reported after ingestion of only 10 g [39, 40]. Finally, recent evidence has suggested that prolonged therapeutic use of APAP may increase the risk for toxicity [41, 42]. Additional studies are necessary to determine the effects of chronic use of APAP and other nephrotoxic drugs on the kidney in elderly human subjects.

Another important aspect of this study was the finding that Cys levels were diminished in the aging kidney and paralleled those of GSH throughout the entire life span. Although we previously found an aging-specific loss of Cys in an aging mosquito model [43], decreases were not observed in other tissues of the aging C57BL/6 mouse [12, 13]. Since the kidney contains high levels of the enzyme γ-glutamyl transpeptidase and represents the major site for GSH degradation and subsequent release of Cys, it is likely that the aging-decrease in Cys is a result, rather than a cause of the GSH deficiency.

As the kidney is considered an important supplier of Cys for other tissues, a decreased release of Cys from this organ during aging could result in a slower turnover, and thus a lower availability of Cys for non-renal tissues. This decreased Cys availability may not be reflected in steady-state Cys levels due to the short half-life and low levels of this compound in these tissues.

It has been suggested that the kidney Cys levels observed in our studies are artificially high due to the postmortem conversion of GSH to Cys [44]. This is untrue, for the present results confirm the accuracy of our Cys analyses and clearly demonstrate that such conversion does not occur when samples are maintained at 0°. The Cys values obtained in this study are for kidney cortex and are higher than those for total kidney, indicating that the renal papilla has a relatively low Cys content. Indeed, this is consistent with GSH which is primarily located in the cortex region [6].

The lack of Cys could have a number of important implications. Cysteine is often a rate-limiting amino acid in the synthesis of proteins and, thus, the capacity for protein biosynthesis in tissues may be impaired. Also, the lack may lead to an impairment of GSH re-synthesis, as well as the production of adenosine 3′-phosphate 5′-phosphosulfate (PAPS), and the subsequent detoxification by sulfation in non-renal tissues. The fact that Cys levels are depleted by APAP and not recovered in aging mice is an additional insult, leaving the organisms at an even greater state of vulnerability.

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