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ABSTRACT In the hen, heat stress (HS) disrupts shell calcification and reproductive processes, including hormone synthesis and egg production. Two studies were conducted to investigate palliative effects of exogenous estrogen or dietary vitamin D_3 on Ca homeostasis and reproductive physiology during HS. Study 1: Hy-Line W36 hens were randomly assigned to thermoneutral (TN) or HS treatments and to 1 of 7 estrogen treatments: zero (control) or one Compudose 200 implant given 1, 2, 3, 8, 9, or 10 d before onset of HS. With no implant, HS reduced plasma estradiol (E_2) and total Ca absorbed (CaT) by duodenal cells ($P < 0.05$). In TN hens with implants, plasma E_2 tripled within 24 h ($P < 0.05$) and remained elevated ($P < 0.05$) through d 9. In HS hens with implants, plasma E_2 rose 6-fold ($P < 0.05$) to equal TN+E_2 concentrations and remained elevated through d 10. In TN and HS hens with implants, the rate of Ca absorption (CaTR) and CaT increased dramatically; the responses were quadratic and essentially identical. Study 2: Hy-Line W36 hens were provided diets formulated either according to NRC requirements (NRC, 1994), or with the addition of 22,000 IU/kg vitamin D_3 (+VD hens). A 24-h HS episode was imposed 2 wk after initiation of the dietary regimen. Duodenal samples were collected for Ca absorption assays after the 24-h HS episode. Both CaTR and CaT in +VD hens were approximately 3-fold higher than in hens in the NVD group ($P = 0.102$). The results lead to the conclusion that exogenous estrogen, high levels of dietary vitamin D, or both, before a HS episode, are efficacious in alleviating at least some of the effects of HS and should be further investigated.

(*Key words*: calcium absorption, Compudose 200, estrogen, heat stress)

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INTRODUCTION

High environmental temperatures cause the loss of millions of dollars to the egg industry each year (Roland, 1988; Bell, 1998). The 3 major factors contributing to these losses are reproductive failure (fewer eggs), poor egg quality (soft shells or shell-less eggs), and impaired skeletal integrity of the hen (Scott and Balnave, 1988). Many studies have attempted to characterize the physiological mechanisms of these failures. Of particular interest are heat stress (HS)-induced changes in acid-base status (Mather et al., 1980; Bottje and Harrison, 1986; Marder and Arad, 1989), ionized Ca (Odom et al., 1986), reproductive hormones (luteinizing hormone, progesterone: Donoghue et al., 1989; Novero et al., 1991; estradiol: Mahmoud et al., 1996), and Ca uptake by duodenal cells in vitro (Mahmoud et al., 1996). Calcium has critical and ubiquitous involvement in egg production (Hertelendy and Taylor, 1961) and hormonal regulation; Ca and estrogen are required for synthesis and release of luteinizing hormone and progesterone (Onagbesan and Peddie, 1989). The complex interactions between Ca and estrogen also include estrogen-activation of vitamin D and enhancement of Ca transport from the gut (Bar and Hurwitz, 1979).

Because of these important interactions and because HS has been shown to decrease ionized Ca (Odom et al., 1986; Staten and Harrison, 1987) and all 3 reproductive hormones in blood, a series of studies was conducted to determine whether treatment with estrogen or vitamin D_3 might prove beneficial during HS. Forman et al. (1996), in a preliminary study, found higher plasma estradiol and greater Ca transport in hens that had received exogenous estrogen before a 12-h HS episode (Forman et al., 1996). Novak (1997) found that feeding high levels of vitamin D_3 for an extended period (several months) of thermoneutral (TN) temperatures improved Ca absorption in vitro. The objectives of these studies were to further elucidate and

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Abbreviation Key: CaT = total calcium absorption; CaTR = rate of calcium absorption; E_2 = 17β-estradiol; HS = heat stress; NVD = adequate layer diet vitamin D_3; TN = thermalneutral; +VD = 22,000 IU/kg vitamin D_3.

define the period of efficacy of estrogen implants and to determine whether the high amounts of vitamin D_3, if fed for a short period (several weeks) prior to an acute HS episode would have the same effect on Ca absorption.

MATERIALS AND METHODS

Experiment 1: Estrogen Implants and Duodenal Cell Ca Transport

Birds. Seventy Hy-Line W36 laying hens, 47 to 52 wk of age at approximately 80% production, were used in this study. The hens were housed 5 per cage in an environmentally controlled room at a constant temperature of 22°C (TN) where they were allowed to acclimate for at least 1 wk. At the start of each experimental treatment, the hens were housed individually. They were provided feed (layer diet: 2,947 kcal of ME/kg of feed, 3.8% Ca, 0.5% P, 17.0% CP) and water ad libitum. The photoperiod was 16L:8D. All studies were conducted with approval of the University of Nebraska-Lincoln IACUC.

Experimental Protocol

Estrogen Treatment Groups. Hens were randomly assigned to 1 of 7 groups, 10 birds per group. Groups were specified by treatment, which consisted of a control (no estrogen) or one estrogen implant (Compudose 200 bovine subdermal[3]) per bird given 1, 2, 3, 8, 9, or 10 d before exposure to HS.

Temperature Treatments. After receiving the implant, hens were placed in 1 of 2 environmental temperatures: a constant TN temperature of 22°C or a constant HS temperature of 35°C. Relative humidity was maintained at 50%. In both treatments, the hens were moved individually into the respective environments at 0700 h at the beginning of the trial.

Blood Sampling. Hens were handled individually throughout the experimental period. As each hen entered the experiment (~0500 to 0700 h), the brachial vein was cannulated using xylocaine 5% jelly topical anesthesia[4] with a 20-g × 3.2 cmi V. catheter.[5] The catheter was sutured into the skin to keep it in place throughout the sampling period and capped with polypropylene male plug fittings.[5] Blood samples were drawn at 0600 h, with 3.0 mL sterile syringes and collected into heparinized 15 × 85 mm test tubes.

Plasma was separated from the red blood cells by centrifugation at 2,000 × *g*. Saturated Na citrate was added to the plasma samples (20 μL/mL) to prevent further clotting (Novero et al., 1991). The samples were stored at –20°C until they were assayed for 17β-estradiol (E_2) by RIA validated for chicken plasma at the Animal Sciences Physiology Laboratory, University of Nebraska-Lincoln.

In Vitro Ca Transport. Immediately following the last blood sample collection, all birds from the particular treatment were euthanized by cervical dislocation, and the duodenal loop was excised. In vitro Ca transport was then determined as described by Al-Batshan et al. (1994), with a slight modification of tissue incubation time. Briefly, 4 thin slices (approximately 1.5 cm × 2 mm wide) were taken from the loop and placed in disposable beakers containing 2.0 mL of the following buffer: 140 m*M* NaCl, 4.8 m*M* KCl, 1.2 m*M* KH_2PO_4, 1.2 m*M* $MgSO_4$, and 25 m*M* HEPES; pH 7.40.[6] After incubation for 10 min at 37°C, the assay was started by transferring the tissue to identical beakers containing 2 mL of the above buffer plus 5 m*M* dextrose and 14 μCi of ^{45}Ca. The tissues were incubated for 4 and 9 min at 37°C in a shaking water bath. The reaction was terminated by transferring the slices to beakers containing 4 mL of 300 m*M* mannitol. ^{45}Ca was extracted from the tissue by incubation in 2.0 mL of 2.5% trichloroacetic acid in a shaking water bath at 37°C for 60 min. The tissue samples were then weighed and recorded and the supernatant was poured into 15 × 85 mm test tubes and centrifuged at 500 × *g* for 5 min. One milliliter of each supernatant was then pipetted into a 20-mL scintillation vial, 6 mL of EcoLite[7] scintillation cocktail was added, and the radioactivity of ^{45}Ca was counted on a β-counter.[7] Data were calculated as the rate of Ca transport (CaTR), that is, the amount of radiolabeled calcium absorbed by duodenal tissue per minute between the 4- and 9-min incubation periods; or total Ca transport (CaT), that is, the amount of radiolabeled Ca absorbed by duodenal tissue during the 9-min incubation period.

Experiment 2: Dietary Vitamin D_3 and Duodenal Ca Transport

Birds. Twenty Hy-Line W36 laying hens, 40 to 46 wk of age at approximately 80% production, were used in this study. The hens were housed 5 hens per cage in an environmentally controlled room with constant temperature of 22°C (TN) where they were allowed to acclimate for at least 1 wk. At the start of each experimental treatment, hens were housed individually. Hens were provided feed (layer diet: 2,947 kcal of ME/kg of feed, 3.8% Ca, 0.5% P, 17.0% CP) and water ad libitum. The photoperiod was 16L:8D. Ten hens were randomly assigned to 1 of 2 dietary treatments, as follows: 1) a normal layer diet with 2,570 IU of vitamin D_3/kg of feed (NRC, 1994) and 2) a layer diet with added dietary vitamin D_3 (22,000 IU/kg; Novak, 1997). Birds were fed the respective diets for 2 wk before initiation of the 24-h temperature treatment, which consisted of a constant HS temperature of 35°C and 50% RH. Hens were moved individually into the HS environment at 0700 h on the morning the trial was initiated. At the end of 24 h, birds were euthanized by cervical dislocation, and the duodenal loop was excised. Ca absorption was determined as described previously.

[3]Elanco Animal Health, Indianapolis, IN.
[4]Astra Pharmaceutical Products, Inc., Worchester, MA.
[5]Baxter Healthcare Corp., Deerfield, IL.
[6]ICN Pharmaceuticals, Inc., Costa Mesa, CA.
[7]Packard C1900 liquid scintillation analyzer, Packard Instrument Co., Meriden, CT.

Statistical Analysis

All experiments were conducted as a completely randomized design, with temperature and implant or dietary treatment as fixed effects and individual cage (hen) as the experimental unit. Plasma estradiol data and all data from study 2 were analyzed by ANOVA using proc GLM of SAS software (SAS Institute, 2000); the CaTR and CaT data in study 1 were analyzed by linear regression implemented in proc mixed of SAS software. The differences among means were determined using the least significance difference test.

E_2 Validation

Radioimmunoassay for E_2 was validated as follows for chicken plasma at the Animal Sciences Physiology Laboratory, University of Nebraska. Duplicate aliquots (6.6 μL) of sample were extracted twice with 2 mL of diethylether, and extract residues were subjected to E_2 RIA as described by Kojima et al. (1992). The assay utilized an antiserum to E_2 at a dilution of 1:1,600,000 (Lilly lot #022367) provided by N. R. Mason.[8] The assay for E_2 was validated as follows. Pooled avian plasma samples (n = 4) were assayed at 100, 50, 25, and 12.5 μL. Four pools of avian plasma were used to determine recovery of added E_2 (0.2, 1.6, and 12.8 pg). Recovery ranged from 76.1 to 111.8%, averaging 87.85 ± 10.65%. Parallelism was determined by using the Allfit program (DeLean et al., 1978). Slopes of the dilutions of plasma and the standard curve were not different as determined by the Allfit program ($P = 0.2610$). The intra- and interassay coefficients of variation were 1.05 and 5.38%, respectively.

RESULTS

Experiment 1: E_2 Implants and Duodenal CaT

Plasma estradiol in HS hens receiving no supplemental E_2 was depressed to half that of control hens at TN conditions ($P < 0.05$; Table 1). In TN hens treated with Compudose 200 implants, plasma estradiol was 3 to 6 times higher than in the nonimplanted (control) birds (Table 1) through d 9; the estradiol concentration on d 10 in plasma from implanted TN hens was not different from that of control TN birds ($P > 0.10$). Essentially the same pattern was observed in HS hens; however, the concentration on d 10 (861 pg/mL) was more than 3 times that of HS hens without E_2 (276 pg/mL; $P \leq 0.05$) (Table 1).

The initial (0) values obtained for CaTR (Figure 1) and CaT (Figure 2) were not statistically different ($P = 0.349$ for CaTR and 0.1275 for CaT) in HS and TN birds, even though the HS values in both cases were considerably lower than those in TN hens— reductions that were most likely biologically relevant. Estrogen implants resulted in significant ($P \leq 0.001$) quadratic responses in TN and HS birds, and there was no interaction of thermal and estrogen treatments; thus, the main effects were evaluated independently. In TN hens, the effect of E_2 implants on CaTR and CaT was not significant except on d 8 and 3, respectively, after they were implanted, even though both began to increase immediately. In HS hens, in contrast, the effect was much more dramatic, with CaTR and CaT increasing ($P \leq 0.05$) on the first day of E_2 exposure and remaining elevated through d 10.

TABLE 1. Plasma estradiol concentrations

Days of estrogen exposure prior to 24-h HS[1]	Estradiol[2] (pg/mL)	
	TN[3]	HS[4]
0	543.90 ± 260.73[a]	276.46 ± 201.96[b]
1	1,536.87 ± 201.96[c]	1,298.72 ± 201.96[c]
2	1,998.01 ± 201.96[c]	1,579.85 ± 201.96[c]
3	962.42 ± 201.96[a,c]	1,096.17 ± 201.96[c]
8	1,410.31 ± 201.96[c]	949.98 ± 225.80[c]
9	1,006.49 ± 319.33[c]	914.76 ± 260.73[c]
10	867.30 ± 260.73[a,c]	861.00 ± 260.73[c]

[a–c]Means ± SEM within row and column without common superscripts differ significantly ($P \leq 0.05$).

[1]Days of treatment with Compudose 200 estrogen implant before 24-h heat stress (HS) episode.

[2]Plasma estradiol concentrations in laying hens.

[3]Thermoneutral conditions, 22°C.

[4]Heat stress conditions, 35°C.

Experiment 2: Dietary Vitamin D_3 and Duodenal CaT

Feeding very high levels of vitamin D_3 for 2 wk before a 24-h HS episode had marked effects on CaTR ($P = 0.1019$; Figure 3) and CaT ($P \leq 0.05$; Figure 4). Although the CaTR increase was not as dramatic, total CaT (Figure 4) by duodenal cells from the +VD hens was elevated to approximately twice that of NVD/HS hens (2,055 vs 1226 nmol/g; $P < 0.05$).

DISCUSSION

In hens maintained at TN, the increase in circulating E_2 from approximately 500 pg/mL to 1,500+ pg/mL was as expected. It has been shown consistently that providing E_2 via Compudose implants elevates plasma estradiol (Qin et al., 1993; Qin and Klandorf, 1995; Forman et al., 1996). When data were examined day by day, the elevation of plasma E_2 in Compudose 200-treated TN birds was slightly less well maintained than in HS birds. However, this finding may be attributable to the higher initial concentration of E_2 in plasma in TN birds.

There was no interaction of HS and E_2 treatment ($P > 0.10$), indicating that treatment with E_2 had a similar effect in both TN and HS birds. Because nonimplanted HS birds had considerably less Ca absorption than nonimplanted TN birds, the response to implants was much more

[8]Lilly Research Laboratories, Indianapolis, IN.

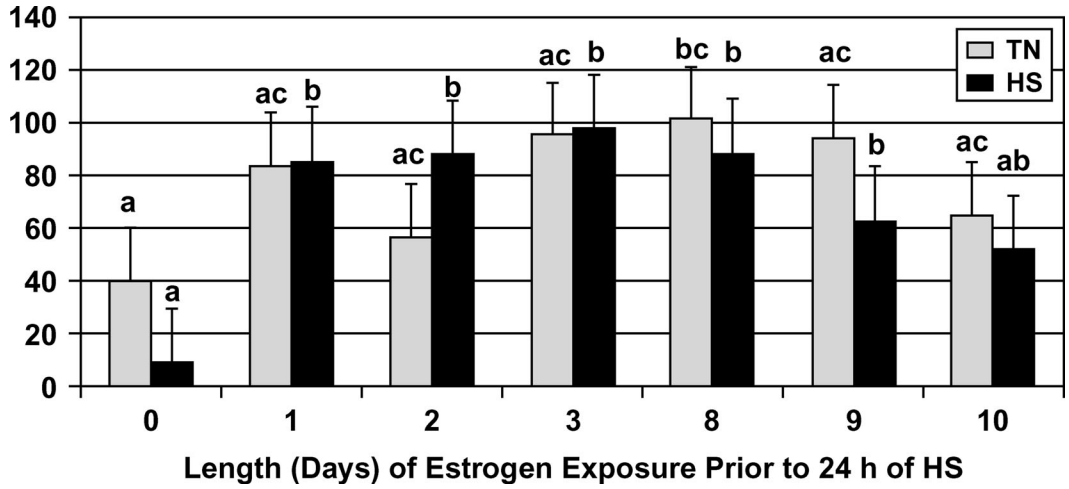

FIGURE 1. Rate of Ca transport in the duodenum of laying hens. Amount (nmol) of radiolabeled Ca absorbed per minute per g of duodenal tissue in vitro of laying hens treated with Compudose 200 implant 1, 2, 3, 8, 9, or 10 d prior to exposure to a thermoneutral (TN) or 24-h heat stress (HS) episode. There was no interaction of HS and estrogen (E_2) treatment. The main effects of HS and E_2 treatment were quadratic ($P \leq 0.05$). Within day (length of exposure to E_2), means were not different; within thermal treatment, means with unlike superscripts were different ($P \leq 0.08$, TN) or ($P \leq 0.10$, HS).

marked in HS than in TN hens. In addition, E_2 implanted in hens up to 10 d before an HS episode proved efficacious in maintaining elevated CaTR and CaT.

Although not addressed in the present study, one plausible hypothesis for the initial benefit of exogenous E_2 to Ca transport would be an increase in parathyroid hormone receptors leading to increased 1α-hydroxylase activity, vitamin D_3 synthesis, and increased Ca-binding protein calbindin D28K (CaBP D28K) in the intestine (Forte et al., 1983; Theofan et al., 1986, 1987; Klandorf et al., 1992; Elaroussi et al., 1993). Supplying excess E_2 for longer than 9 d could have resulted in one or more of these mechanisms reaching a maximum level and subsequently showing no further improvement. Alternatively, E_2 has been shown to increase blood flow to various reproductive organs (Rosenfeld et al., 1976; Wiltbank et al., 1989). Considering this, estrogen may influence both the availability of Ca^{2+} for cellular processes and the chemical gradient. Thus, the failure to improve CaTR or total CaT after 9 d could be the result of a readjustment in the ionic gradient. Finally, saturation of estrogen or parathyroid hormone receptors in the kidney or estrogen receptors in

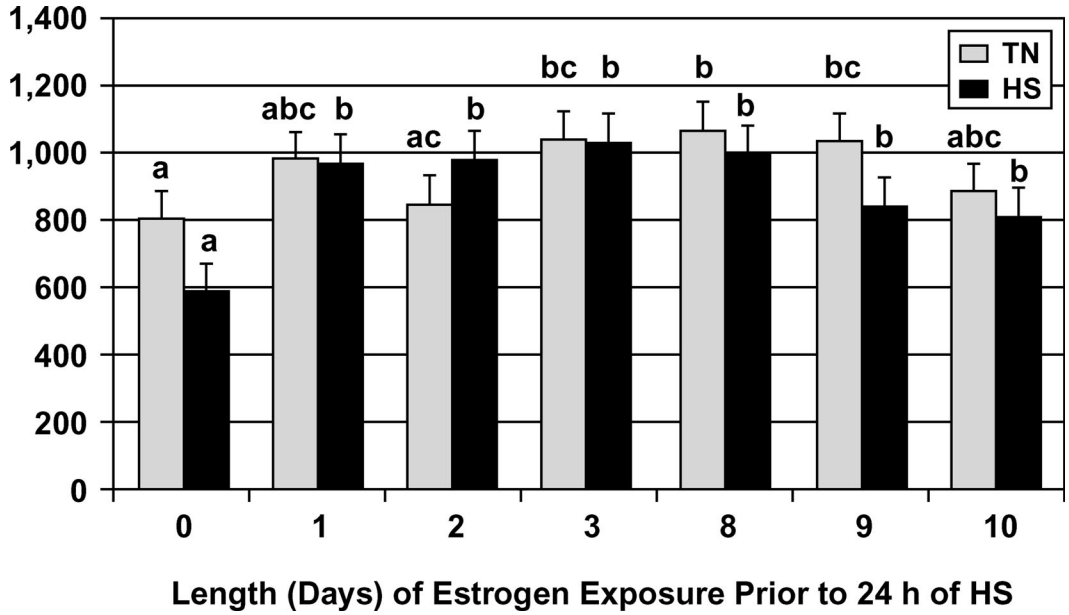

FIGURE 2. Total Ca transport in the duodenum of laying hens. Amount (nmol) of radiolabeled Ca absorbed by duodenal tissue in vitro of laying hens treated with Compudose 200 implant 1, 2, 3, 8, 9, or 10 d prior to exposure to a thermoneutral (TN) or 24-h heat stress (HS) episode. There was no interaction of HS and estrogen (E_2) treatment. The main effects of HS and E_2 treatment were quadratic ($P \leq 0.05$). Means within days were not different; within thermal treatments, means with unlike symbols are different ($P \leq 0.1$, TN) or ($P \leq 0.079$, HS).

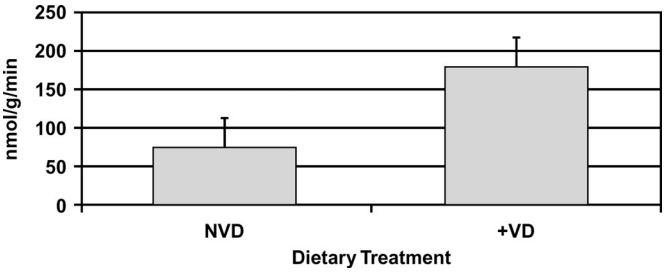

FIGURE 3. Rate of Ca absorption in duodenum of laying hens following 1 d of heat stress (HS). Amount (nmol) of radiolabeled Ca absorbed per minute per gram of duodenal tissue in vitro of laying hens fed adequate (NVD) or greater than adequate (+VD) vitamin D_3 and exposed to a 24-h HS episode. The rate of Ca absorption in + D-hens was ~3-fold higher than in hens in the NVD group ($P = 0.1019$).

the gut could have resulted in the cessation of active vitamin D_3 production and may have served as a causative or contributory factor in the failure of Ca transport to remain elevated.

In the second study, the addition of 22,000 IU of vitamin D_3/kg of feed—an order of magnitude above normal—appeared to alleviate the effects of acute heat stress by significantly improving calcium absorption. Vitamin D_3 is a sterol hormone and, therefore, is likely to remain hormonally active when ingested orally. Administration of 1,25-dihydroxycholecalciferol via the diet essentially places the active hormone at the site (the intestine) where it can exert an effect on Ca absorption as an intact, activated steroid hormone. Vitamin D_3 is necessary for the production of calbindin D28K in the intestine (Theofan et al., 1986); therefore, if this hormone remains in its active form, excess dietary vitamin D_3 may increase calbibdin D28K synthesis, thus increasing calcium absorption at the intestine.

In a preliminary study, Novak (1997) found that greater than typical amounts of dietary vitamin D_3 fed for a long (3 to 4 mo) improved Ca transport under TN conditions. In the present study, the dietary treatments were short term (2 wk), but the elevation of Ca absorption was comparable with that of the longer study (Novak, 1997). The elevation of Ca absorption in hens subjected to acute HS episodes was even more marked than in hens at TN

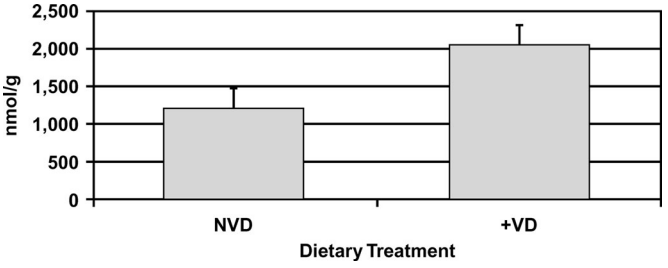

FIGURE 4. Total Ca absorption (CaT) in duodenum of laying hens. Amount (nmol) of radiolabeled Ca absorbed by duodenal tissue in vitro of laying hens fed adequate (NVD) or greater than adequate (+VD) vitamin D_3 and exposed to a 24-h heat stress episode. CaT was enhanced by +VD ($P \leq 0.05$).

conditions, leading to the suggestion that 22,000 IU/kg vitamin D_3 could have potential as a management tool if fed to birds prior to a predicted HS period. Further studies are required, and are underway, to substantiate this and to determine the optimum timing interval between feeding excess vitamin D_3 relative to an expected HS episode. It will also be important to determine whether improved in vitro CaT correlates with improved shell or bone calcification during periods of high environmental temperatures, because cells acting outside of the intact animal may well respond somewhat differently than in an intact system in the presence of all of the available factors.

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