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Infrared Radiation Influence on Molt and Regeneration of *Neohelice granulata* Dana, 1851 (Grapsidae, Sesarminae)

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ABSTRACT

This paper analyzes the influence of infrared radiation (IR) on regeneration, after autotomy of limb buds of *Neohelice granulata* and consequently the time molt. Eyestalks were ablated to synchronize the start of molt. Afterward, animals were autotomized of five pereopods and divided into control and irradiated groups. The irradiated group was treated for 30 min daily until molt. Limb buds from five animals of days 4, 16 and 20 were collected and histological sections were made from them. These sections were photographed and chitin and epithelium content measured. Another group was made, and after 15 days limb buds were extracted to analyze mitochondrial enzymatic activity from complex I and II. The irradiated group showed a significant reduction in molt time (19.38 ± 1.22 days) compared with the control group (32.69 ± 1.57 days) and also a significant increase in mitochondrial complex I (388.9 ± 27.94%) and II (175.63 ± 7.66%) in the irradiated group when compared with the control group (100 ± 17.90; 100 ± 7.82, respectively). However, these effects were not acompanied by histological alterations in relation to chitin and epithelium. This way, it was possible to demonstrate that IR increases complex I and II activity, reduces the time molt and consequently increases the appendage regeneration rate.

INTRODUCTION

Autotomy is a process that some animals perform to detach a body part, as a defense mechanism against predation (1). During this process, quick internal fluid loss is prevented (2). Particularly, in decapod crustaceans, each appendage has a single break plane, located next to its base. In brachyurans, such the *Neohelice granulata* crab, this break line is next to the fusion region of the basiopodite with the ischiopodite, resulting in good autotomy capacity. However, even with the disadvantages that come with the loss of a member, they are all balanced by a well-established interaction between autotomy and molt (ecdysis) (1).

Regeneration occurs even in adults and, unless the *coxa* has been removed or injured, this regeneration is morphologically complete at the first ecdysis, although the appendage has less mass than its not-regenerated conterlateral (3).

Autotomy and ecdysis are influenced by the environment, such as food availability needed to cell division process and/or competition with other living beings. These biological factors can be handled to some degree by the individuals. Abiotic factors also interfere with autotomy and ecdysis. Among these abiotic factors, infrared radiation (IR) is worth noting, known for its capacity to stimulate cellular proliferation in mammals (4).

Vertebrates do not perceive it as light (an exception is the snake, which has a nonocular visual system) but as heat, by skin specialized nervous ends, known as thermoreceptors (5). IR is arbitrarily divided into three categories: near-IR (0.7–1.5 μm), middle (1.5–5.6 μm) and far (5.6–1000 μm). At Rio Grande do Sul, Brazil, its medium value for the year 2006 was 408.8094 W m^{-2} (6). Older studies showed differences between biological action of near-IR and middle/far IR (7). It was believed that near-IR penetrated equally to a deeper portion of the skin without causing any temperature increase on the skin's surface, while the largest part of middle/far IR energy was absorbed by the skin's superficial layer and frequently caused harmful thermal effects such as thermal burns or burning.

Middle/far IR as all IR wavelengths have cellular regenerative effects (8–10). *In vitro* studies with near-IR on human endothelial and keratinocytic cells showed an increase in transforming factor-β1 (TGF-β1) and matrix metalloproteinase-2. These two proteins are involved in the wound repair–remodeling phase. These effects were considered athermic as the models used as thermal control showed no increase in their protein expression.

Experiments with far IR showed tumor growth inhibition in mice and clinical improvement in scabs. Irradiation also showed improvement in the regenerative process of mice without any concomitant increase in blood circulation or skin temperature. Other data demonstrate an increase of infiltration fibroblasts in the subcutaneous tissue of mice treated with far

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IR and higher collagen regeneration on the wounded region. Far-IR also increased TGF-β1 expression. IR increased angiogenesis in the wound region and tensile strength in the regenerative skin (11–13). In contrast to the initial idea that far IR had deleterious effects, it is believed nowadays that its biostimulatory action equals that of low-potency lasers and near-IR (14).

The basic premise is that electromagnetic radiations of long wavelength stimulate energetic metabolism of cells, such as energy production. There are three IR photoacceptor molecules in mammals known to absorb near-infrared wavelength: hemoglobin, myoglobin and cytochrome *c* oxidase. From these photoacceptor molecules, it is believed that mitochondrial photoacceptors must be responsible for 50% of the near-infrared absorption, through cytochrome *c* oxidase (4,15,16).

All experiments with near-IR or middle-far IR were performed on vertebrates. Consequently, there is no information about the effects of IR on invertebrate regeneration, and in direct relation, there are no known effects on the molt cycle.

Of the three aforementioned photoacceptor molecules, cytochrome *c* oxidase is present in crustaceans. Hemoglobin is substituted by hemocyanin as the gas carrier pigment and there are no data of its infrared bioactivation.

The crustacean, *Neohelice granulata* Dana, 1851 (Grapsidae, Sesarminae), is a semiterrestrial crab characteristic of Lagoa dos Patos, an estuarine environment. It can be found in the south and southeast coast of Brazil and in Uruguay and Argentina. There is an extensive bibliography on the habits and ecology of this crab, but little information on its regenerative process or its autotomy. As it is a brachyuran, it can be assumed that the appendage break plane is fairly developed, and on this basis, that the animal has a good autotomy capacity. Its small size, even when adult, facilitates easy handling and exposition of the entire body to IR (17).

The aim of this study was to determine the IR effects on molt and regeneration time of *N. granulata* besides analyzing any histological changes at the limb bud.

MATERIALS AND METHODS

Capture and acclimation. *Neohelice granulata* specimens were collected at the estuary near Cidade de Rio Grande, RS, Brazil (S 32°09.031′, W 52°06.151′). They were maintained in tanks with 20 salinity water, 20°C room temperature and 12:12 L/D photoperiod, after a week of acclimation. During acclimation and experiments, animals were fed *ad libitum* with bovine meat.

Infrared lamp and irradiation procedure. All irradiations were made with a Phillips Infrared lamp (Infraphil Healthcare 13379F/479) with wavelength ranging from 0.6 to 1.5 μm and peak at 1.0 μm (near-infrared lamp). The 150 W lamp was kept 30 cm over the bottom of the aquarium. Water temperature was measured, after irradiation, at the bottom of the aquarium and at the water lamina. These measures were in agreement with the 20°C of room temperature. Within these distances, there was no water heating. So, all experiments were made without water temperature change when compared with the control group. For irradiation, animals were kept in an aquarium with 28 cm × 16 cm × 12 cm (length, width, height), filled with 4.3 L of 20 salinity water, for 11 cm of water column. Exposition time for all experiments was 30 min. All animals had an average area of 6.25 cm^2, and exposition dose was of 41.5 J cm^{-2}.

Critical autotomy number evaluation for molt—experiment A. To determine the optimal number of pereopods that would be autotomized by mechanical traction, we divided eight groups (n = 4 each). Animals were grouped by number of autotomized appendices: group 1 had one appendage autotomized, group 2 had two appendices autotomized from each side, and so on until group 8. Appendices were autotomized to strike a balance on both sides of the animals. To prevent hemolymph loss when animals were put back in the aquarium, they were maintained outside water for 30 min, allowing the formation of a clot. Each animal was observed and the time to molt (in days) was verified. The experiments were replicated.

Autotomy and infrared exposition—experiment B. Groups of 10 animals were made until control and irradiated groups had 32 animals each. The animals were ablated and autotomized of five pereopods (two from one body side and three at the other). The irradiated group was treated with IR for 30 min daily (same exposition time used in vertebrate experiments) starting after autotomy, until all animals had molted. Thereafter, molt animals were killed by freezing at 0°C for 1 h. All groups were fed *ad libitum* with bovine meat.

Histological analysis of limb buds—experiment C. In this analysis was chosen the chitin and the epithelium content of the limb bud (irradiated and control). Both groups had 20 animals and five pereopods were autotomized in each animal. The irradiated group was treated with IR for 30 min, per day, to a maximum of 20 days. Both groups received food *ad libitum*.

Days 4, 16 and 20 were chosen for evaluation, day 4 being the start of the regeneration period and days 16 and 20 the end of the regeneration period. On days 4, 16 and 20, four animals from the control group and four from the irradiated group were killed by freezing at 0°C for 20 min. Their limb buds were then surgically removed, for a total of 20 limb buds, per group per day. The limb buds were prepared as described by Borges (18) as follows: each limb bud was fixed in Davidson solution for approximately 2 weeks. To assess the decalcification of the limb bud, the oxalate test was used (5 g solution of sodium oxalate in 100 mL of distilled water). This analysis was made using 5 mL of Davidson solution from the fixation piece, mixed with 1 mL of oxalate. After decalcification, limb buds were dehydrated with successive concentrations of alcohol 70%, 80%, 90%, 96%, 100% I, 100% II for 30 min in each alcohol, diafanated in Xilol I and Xilol II for 15 min each, included in paraffin I and II for 15 min and finally on paraplast, after each limb bud was microtomized at 7 μm thickness. Each cut was perpendicular to chitin and epithelium content. Three preparations were made with each included limb bud (n = 60) and stained with Mallory Trichromium.

Pairs of control and irradiated laminas were photographed with a Sony DSC-H1 camera using an Olympus microscope with 10× ocular and 40× objective. Photographs had 7 megapixel of resolution. Along with every pair of photograph, a 1 mm control lamina was photographed and used as a control for measurement. Photographs were measured for chitin and epithelium length content using AxioVision LEV 4.6.3.0 from Carl Zeiss Imaging Solutions. AxioVision was calibrated using the control lamina. Each sample photograph had three random linear measures made on it for chitin and for epithelium, totaling 180 measures for each evaluation day to irradiation and 180 measures to control.

Mitochondrial analysis—experiment D. To determine whether IR was affecting the energy production of irradiated animals, we analyzed the activities of mitochondrial respiratory chain enzymes of complex I and II in relation to the total mitochondrial protein content. Animals had five pereopods autotomized and had their eyestalks ablated. They were divided into two groups, irradiated and control, both with 20 animals each. The irradiated group received 30 min daily of IR. Animals received food *ad libitum* and were maintained in aquariums with 20 salinity water at 21°C room temperature. After 15 days, all surviving animals were killed, making for five irradiated animals, each with four limb buds for analyses (n = 20) and four control animals each with four limb buds for analyses (n = 16). Limb buds were removed and maintained on ice during analysis.

Mitochondrial isolation was made as described by Birch-Machin *et al*. (19). Limb buds were homogenized with 10 mL of isolation buffer (530 mM sucrose, 200 μM EGTA, 1 mM EDTA, 20 mM HEPES, 0.5% BSA, pH 7.6) per tissue gram and the resulting mixture was centrifuged at 1600 ***g*** for 15 min at 4°C. The supernatant was removed and centrifuged at 7100 ***g*** for 15 min at 4°C. The pellet was maintained on isolation buffer. Thereafter, it was resuspended in 200 μL of respiration buffer (303 mM sucrose, 90 mM KCl, 1 mM EGTA, 4 mM KH_2PO_4, 20 mM HEPES, 0.5% BSA, pH 7.5) and maintained on ice for further complex analysis.

Complex I (NADH-dehydrogenase) was evaluated by oxidation of NADH by NADH dehydrogenase at λ = 340 nm. The samples were dissociated mechanically with a syringe and then 10 μL of sample was mixed with 25 μL of NADH (200 μM) and completed to 500 μL with TFK (pH 7.4). Thereafter, the samples were analyzed on a spectrophotometer for 3 min to calculate the enzyme kinetics.

The activity of succinate-2,6-dichloroindophenol-oxidoreductase (complex II) was evaluated by MTT (3 (4,5-dimethilthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazol) reduction at λ = 570 nm. Dissociated samples (50 μL) were mixed with 10 μL of succinate (5 mM), 300 μL of isolation buffer and 10 μL of MTT (5 μg mL^{-1}) and incubated for 15 min. After incubation, 1 mL of DMSO was added and left to react for 15 min. Total protein content in samples was determined using a reagent kit based on the Biuret assay (Doles, Goiânia, GO, Brazil).

Activities of enzymes involved in mitochondrial respiratory chain complexes (I and II) were calculated as absorbance units min^{-1} mg^{-1} protein. Changes in enzyme activity were calculated as percentage considering the control value as 100%.

Statistical analysis. Data were expressed as mean (± standard error). Experiment A was analyzed by ANOVA followed by the Tukey multiple range test. Experiments B and C were analyzed by paired sample *t*-test. Experiment D was analyzed by Student's *t*-test. Significance level was fixed at 0.05.

RESULTS

Experiment A

All groups molted within 60 days of the experiment. As shown in Fig. 1, some autotomized groups were statistically significant, as indicated by different letters. So animals with three, four, five and six appendages autotomized were not different ($P > 0.05$) from each other, but different ($P < 0.05$) from animals with one, two, seven and eight appendages autotomized. Animals with one and eight appendages were similar to each other, but different from other groups. Animals with two autotomized appendages were statistically similar to animals with seven and eight appendages autotomized, and were different from all other groups. Animals with five autotomized appendages had the least number of days until molt and that was significantly different in relation to animals of one, two, seven and eight autotomized appendages, as shown in Fig. 1. With this result, all animals for further assays had five pereopods autotomized.

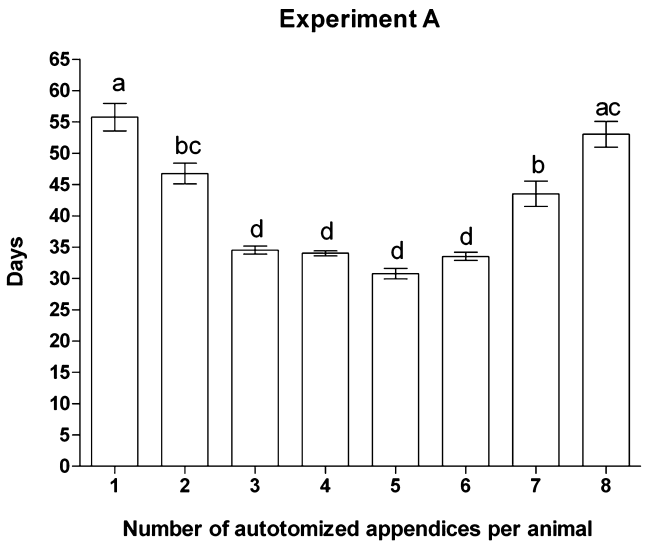

Figure 1. Days until molt from animals with one to eight appendages autotomized. Data are expressed as mean ± SEM. Rows with similar letters indicate the absence of significant differences ($P > 0.05$), so that row 1 is similar to row 8, but different from all the other rows.

Experiment B

Figure 2 shows that irradiated animals had a mean molt time of 19.38 (±1.22) days which was statistically significant against the control group (32.69 ± 1.57) ($P < 0.05$). After molting, neither appendages nor the whole body of animals had morphological alterations in each group (data not shown).

Experiment C

As shown in Figs. 3 and 4 there was no statistical significance in chitin or epithelium content length between the control and irradiated groups for 16 and 20 day animals of experiment. Besides, histological analyses from 4 day irradiated or control animals showed only the autotomic membrane, without any statistically significant difference (data not shown).

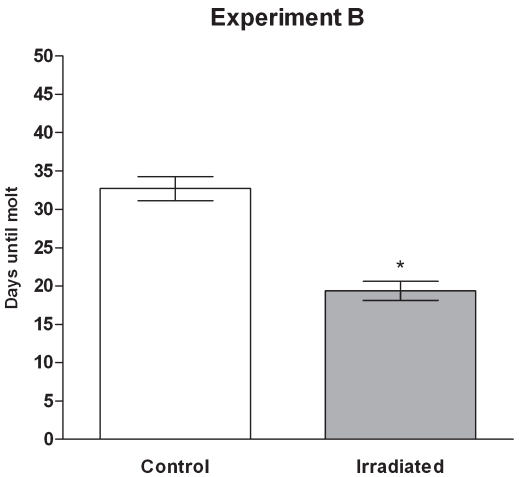

Figure 2. Days until molt of animals treated with 41.5 J cm^{-2} infrared (irradiated group) and of control group (without irradiation). Data are expressed as mean ± SEM. *Significant differences ($P < 0.05$).

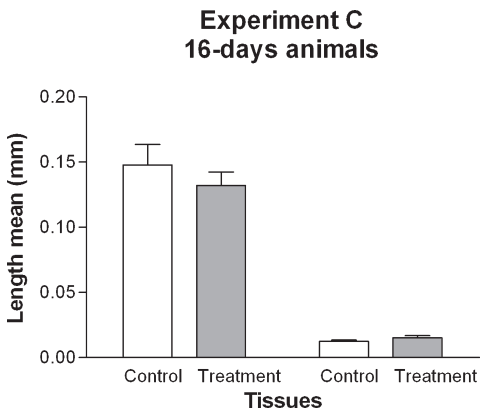

Figure 3. Chitin and epithelium length content of animals treated with 41.5 J cm^{-2} infrared (irradiated group) and of control group (not irradiated) for 16 days. Data are expressed as mean ± SEM. No statistical difference ($P < 0.05$).

Experiment D

As shown in Fig. 5, the irradiated group had a significant increase in the mitochondrial complex I activity (388.9 ± 27.94%) in relation to the control group (100 ± 17.90%). A similar increase was observed for complex II—the irradiated group had a mitochondrial enzyme activity of 175.63 ± 7.66% when compared with the control group (100 ± 7.82%).

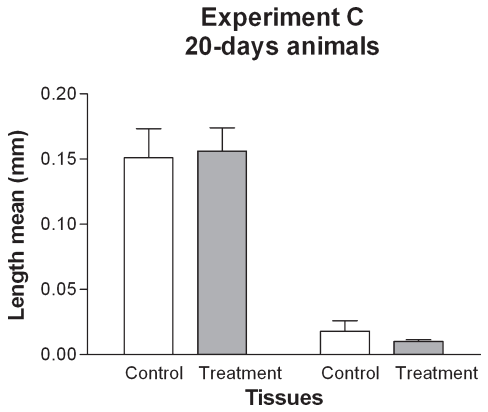

Figure 4. Chitin and epithelium length content of animals treated with 41.5 J cm^{-2} infrared (irradiated group) and of control group (not irradiated) for 20 days. Data are expressed as mean ± SEM. No statistical difference ($P < 0.05$).

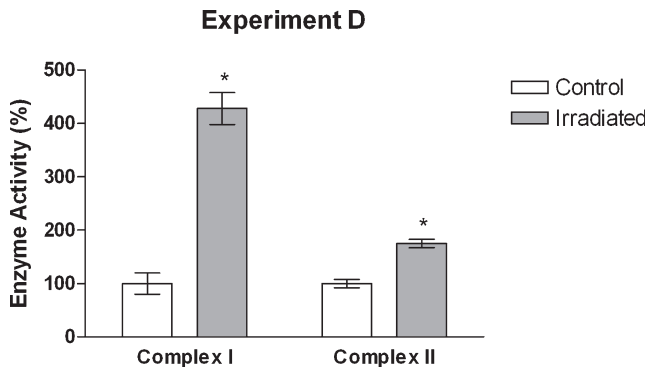

Figure 5. Activities of the complex I and II enzymes of the mitochondrial respiratory chain in *Neohelice granulata* limb buds after 15 day experimentation. Values are expressed as mean ± SEM ($n = 5$ irradiated, 4 control). *Significant differences ($P < 0.05$).

DISCUSSION AND CONCLUSION

Autotomy is a survival ecological strategy. When fighting, *N. granulata* (Fig. 6A) get rid of the appendage and escape the dangerous situation. This loss will cause a hindrance to movement and the need to regrow the appendage. Animals have to balance the cost of all molt process or the regenerative load (maximum of tissues that can be synthesized on the time available to molt) as proposed by Skinner (3) with their food uptake. In experiment A, animals with one, two, seven and eight appendages autotomized had longer molt times than animals with three, four, five and six appendages autotomized. This could be caused by high metabolic cost or the postponed need of appendage recovery. Animals with low appendage loss have little need to regenerate them because their survival is not hindered. So molting would imply an energy cost higher than their actual need of food uptake, assuming that the regenerative load can be achieved with a higher time of tissue synthesis. Besides, animals with seven and eight autotomized appendages must molt rapidly to recover their full survival capacity, so all energy available (not used on basal metabolism) is channeled to the regeneration of the appendages. However, the energy to grow *de novo* seven to eight appendages could not be achieved fast, even with daily *ad libitum* food. This way, the energy uptake they had was lower than their actual need to regrow the appendages in a shorter time. Animals with three to six appendages autotomized had a need to molt, with energy cost and food uptake at balanced levels. The animals with five autotomized appendages presented lower molt time.

When five autotomized appendage animals (Fig. 6B) were submitted to IR, they molted earlier than control animals. Between the known chromophores for IR, only cytochrome *c* oxidase is found in crabs (16). The other two chromophores for IR are hemoglobin and myoglobin, which *N. granulata* do not have.

This absorption probably increased the ATP output, because IR stimulates electron transfer at cytochrome *c* oxidase (20). As all experiments were performed without changing the temperature, it is evident that the stimulation and reduction in time molt (13 days or 40.6%) are due to the biostimulatory effects of IR in increasing ATP production by mitochondria and not to IR heat (5). The data also show that IR has similar effects on this species of invertebrate and on mammals, with the exoskeleton not being a barrier for IR wavelength. There was no effect on chitin deposition or on epithelium bud growth (Fig. 3).

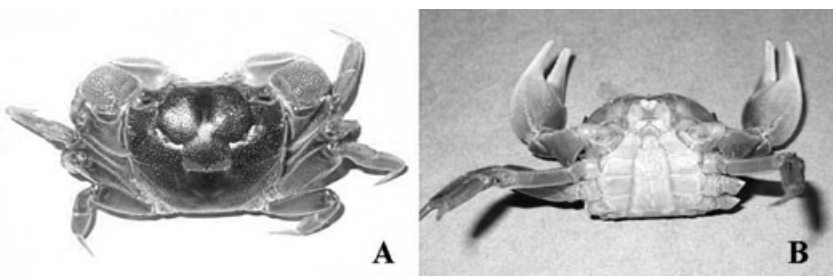

Figure 6. (A) Dorsal view of *Neohelice granulata* before autotomy of pereopods and ablation of eyestalks. (B) Ventral view of *N. granulata* with five pereopods autotomized.

The choice of these parameters (chitin and epithelium) was based on the fact that both are components of the tissues that surround the highly proliferative environment inside the limb bud. Although these tissues were the first structures to be directly exposed at IR, it is confirmed that the whole body was irradiated. So, the metabolism of all tissues of the animals would be higher, although not confirmed by histological analysis. What could have been responsible for the earlier molt in the irradiated animals needs to be determined.

The results suggest that possible alterations have occurred at the molecular level. To confirm the hypothesis that IR increases ATP output, experiment D was performed. This assay analyzed the activity of complex I (NADH: ubiquinone oxidoreductase) and II (succinate: ubiquinone reductase) enzymes of the mitochondrial respiratory chain. Results obtained showed an increased activity of complex I and II enzymes in irradiated crabs.

Complex I is the largest of the mitochondrial respiratory chain enzymes. It actively transports protons from the matrix to the cytoplasmic side of the inner membrane. These create a proton gradient in the cytoplasmic side (21). Complex II transfers electrons from succinate to the ubiquinone pool, feeding electrons to the electron transport chain. But as complex II contains FAD it does not translocate protons to the cytoplasmic side of the inner membrane (22). The electrons passing between the mitochondrial complexes release energy from the proton gradient, and this is used by ATP synthase to make ATP from ADP (21–23).

Complex I and II are part of the oxidative phosphorylation process from the mitochondrial respiratory chain, and most of the cell energy is obtained through oxidative phosphorylation. IR increases the activity of complex I and II, as shown in this work, and of complex IV (4,15,16). An increase in the activity of these complexes reflects an increase in ATP production.

A possible explanation for the higher activity of complex I in relation to complex II in experiment D is that cellular respiration produces more NADH + H (used in complex I) than $FADH_2$ (used in complex II) (24). Therefore, it was expected that the activity of complex I enzymes would be higher than those of complex II. This finding indicates that IR activates not only cytochrome *c* oxidase (complex IV) but also enzymes of complex I and II.

In this way, the results for both complexes show an increase in their enzyme activity in relation to control, confirming that even in invertebrates IR stimulates the increase in mitochondrial enzyme activity. This increase may explain the time reduction during molting.

This study on *N. granulata* demonstrated that IR causes no alteration in the chitin and epithelium content of the limb bud but reduced the molt time that can be related to the higher mitochondrial enzyme activity. Also, it demonstrated that IR enhances the mitochondrial complex I and II enzymatic activity. Besides, it is possible to suggest that the exoskeleton does not hinder the action of IR.

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