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Immunological indices of Giant African Land snails (*Archachatina marginata*) improved with fixed dose of vitamin C during acute heat stress

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ABSTRACT. The study determined immunological indices of Giant African Land snail (*Archachatina marginata*) improved with fixed dose of vitamin C under acute heat stress (AHS). Prior to the AHS, vitamin C was administered for four weeks to two treatment groups, while other two treatment groups were not. Each treatment was monitored, haemolymph collected at 0, 30 and 60 minutes exposure times. Immunological cytokines: interferon gamma (IFN-γ) and interleukin 2 (IL-2); and total haemocyte counts (THC) were determined. Under AHS, vitamin C elevated ($p < 0.05$) IFN-γ production (606.33 ± 302.86) compared to other groups with or without vitamin C administration (7.20 ± 1.58 vs. 73.20 ± 32.23 vs. 7.80 ± 1.36). IL-2 was not affected ($p > 0.05$) by vitamin C under AHS. Highest ($p < 0.05$) THC values was obtained with vitamin C administration under AHS, but reduced under no AHS. Exposure time affected ($p < 0.05$) IFN-γ production and THC values, but not IL-2 ($p > 0.05$). With fixed dose of vitamin C and exposure time, highest ($p < 0.05$) IFN-γ values were obtained under AHS with vitamin C administration at 30 minutes and at 60 minutes in THC, compared to other groups. The study concluded that fixed dose of vitamin C at 150 mg kg^{-1} of feed was appropriate under AHS to boost the immune system of the animals.

Keywords: exposure time; immunological cytokines; interferon gamma; interleukin 2; total haemocytes counts; land snails.

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Introduction

The Giant African Land snail (*Archachatina marginata*) is naturally distributed in agricultural areas, moist lands, and forests when the environmental condition is optimum. At hot temperature and reduced relative humidity, environmental conditions become adverse causing heat stress, which pose challenges to the snails (Vogler et al., 2013). The heat stress becomes acute on short exposure or chronic on lengthy exposure of the animal to the adverse environmental conditions. Temperature increase above room temperature (25oC) has been reported to trigger changes in the haemocytes concentration or morphology which may play key role in the health status of the animal (Adema, Harris, & van Deutekom-Mulder, 1992). The environmental stress challenge influences the haemocytes composition and immune status which could result to death of the animal when not well managed.

It is also noticed that when environmental temperature is at extreme, haemolymph immunological integrity is also at risk thereby triggering immunological activities in response to the thermal conditions, which increases the activities of the haemocytes (Fisher, Auffret, & Balouet, 1987). This also immensely influence the immunological cytokines, like interferon gamma (IFN-γ) and interleukins 2 (IL-2); and upsurge total haemocytes counts of the animal to combat the impending danger to the haemolymph immune system. Therefore, these haemocytes immune parameters can be used as biomarkers to assess how environmental condition affects mollusc health status (Chen et al., 2008).

According to Tewary and Patra (2008), vitamin C has beneficial effect which has been established in several animal species, to stimulate the immune response. Research has shown vitamin C to be a crucial player in numerous aspects of the immune system, principally immune cell function (Maggini, Wintergerst, Beveridge, & Hornig, 2007; Webb & Villamor, 2007). Thus, vitamin C has shown to contribute to immune-modulating effects; ability to easily donate electrons, thus protecting important biomolecules from damage by oxidants generated during normal cell metabolism or exposure to environmental stress (Carr & Frei, 1999).

Exposure to extremely high or low temperatures, disrupt rates of metabolic and physiological processes, including the immune system. Thus, mechanisms of stress tolerance in land snail offer fundamental insights into the adaptation of the organisms for a wide range of environmental challenges, regulated by physiological adaptation (Canesi, Gallo, Gavioli, & Pruzzo, 2002) of immune response. Less attention has been given to the immunological thermoregulatory abilities of the Giant African Land snail under extreme thermal conditions; therefore, understanding of the defense mechanisms is necessary to the establishment of adaptation limits and possible preparation in the changing climate for better productivity. Therefore, the study aimed to determine the immunological indices of Giant African Land snails (GALs) improved with fixed dose of vitamin C under acute heat stress.

Material and methods

Experimental site

The experiment was carried out at the Snail Research Unit of the Department of Animal Physiology, College of Animal Science and Livestock Production (COLANIM), Federal University of Agriculture, Abeokuta, in the rain forest vegetation zone of western Nigeria at latitude 7°N, longitude 32°E and altitude 76 m above sea level (Google earth, retrieved on July 10, 2018 from https://www.google.earth). The climate is humid with a mean annual rainfall of 1,037 mm, an average temperature of 34.7°C and an average relative humidity of 82% throughout the year.

Experimental design

A total of forty (40) Giant African Land snails (*A. marginata*) composed of 5 replicates with two animals per replicate were used for the experiment. These comprised each of four (4) treatment groups placed in plastic cages of dimension 30 cm by 40 cm by 24cmusing randomized complete block design (RCBD). The treatment groups are presented as follows;

Treatment 1: No acute heat stress + No vitamin C (nAHS + nVC)
Treatment 2: No acute heat stress + Vitamin C (nAHS + VC)
Treatment 3: Acute heat stress + No vitamin C (AHS + nVC)
Treatment 4: Acute heat stress + Vitamin C (AHS + VC)

Experimental animal and management

The GALs were weighed using a sensitive scale and randomly allocated to the experimental units of the four distinct groups and then kept for two weeks acclimatization in the experimental environment. The animals were placed in the plastic cages containing a drinker and a feeder, cleaned daily to avoid infections. They were fed *ad libitum* with pawpaw leaves and formulated diet as shown in Table 1 below. At the end of the two-week acclimatization period, fixed dose of vitamin C was administered through the feed at 150 mg kg^{-1} concentration *ad libitum* for four weeks to the animals in group 2 and 4, while those in group 1 and 3 were not administered fixed dose of vitamin C.

Table 1. Composition of experimental diet (g 100^{-1} g).

Constituent	Quantity (g)
Maize	50.00
Wheat offal	27.75
Groundnut cake	12.25
Soyabean meal	4.00
Bone meal	3.00
Oyster shell	3.00
Total	100

Calculated analysis of the diet: Energy- 10,015 kJ kg^{-1}, crude protein- 16.40%, ether extract- 4.21%, crude fibre- 4.42%, calcium- 2.37%, phosphorus- 0.70%, ash- 1.50%.

Induction of acute heat stress

To induce acute heat stress in Giant African Land snails (*A. marginata*), a coal pot was used for the experiment with charcoal for the heat generation in closed chamber (room). The hot charcoal pot was homogeneously placed in the room designated for heat treatment with monitoring gadget (digital thermos-hygrometer) previously mounted. The two groups (3 and 4) of GALs for acute heat stress were placed after

the room had attained uniform temperature. They were exposed to acute heat stress at 0, 30 and 60 minutes durations. The groups (1 and 2) that did not receive heat stress were separated to another room. Changes in the temperature and relative humidity of the rooms were monitored using the digital thermos-hygrometer at the different exposure times.

Haemolymph collection

The haemolymph was collected from the animals using 2 mL syringe and needle via the neck region from the 4 treatment groups. Haemolymph was collected from all the groups at 0, 30 and 60 minutes from the snails with or without acute heat stress. The haemolymph collected was transferred immediately to an Eppendorf tube, for storage. The stored haemolymph was used for immunological cytokines and total haemocytes count analyses.

Determination of immunological cytokines
Interferon gamma and interleukin 2

The interferon gamma (IFN- γ) and interleukin 2 (IL-2) parameters were determined using Enzyme Link Immuno-Sorbent Assay (ELISA) kits procedure of Melsin Medical Company Limited, China.

All reagents were prepared (wash solution: dilute with distilled water at 1:20) prior to the assay and it was ensured that all standards and samples were added in duplicates to the micro ELISA strip plate. A 50 µL standard was added to the standard wells, while 10 µL testing samples were added to the testing samples wells and nothing added to blank well. Then, 40 µL sample diluent was added to testing samples well. A 100 µL HRP-conjugate reagent was added to each well, covered with adhesive strip and incubated for 60 minutes at 37^{o}C. Each well was aspirated, the process repeated four times. This was done by filling each well with wash solution of 400 µL using a squirt bottle, auto-washer. The complete removal of the wash solution was ensured by aspiration and decanting. A 50 µL chromogen solution A and 50 µL chromogen solution B were added to each well, gently mixed and incubated for 15 minutes at 37^{o}C. A stop solution of 50 µL was then added to each well and the wells colour changed from blue to yellow. Thereafter, the optical density was read at 450 nm using micro titre plate reader (EMax Plus Microplate Reader, Molecular Devices, USA) within 15 minutes[1].

Determination total haemocyte counts

Total haemocyte counts (THC) was determined by placing 100 µL sample of haemolymph on a haemocytometer and, haemocytes were counted out and expressed as x 10^6 cells mL^{-1} haemolymph according to Hégaret, Gary, and Philippe (2003) procedures.

Statistical analysis

The data collected were subjected to general linear model analysis of variance (ANOVA) in a randomized complete block design using MINITAB 18 statistical software. The significant treatment means were separated using Tukey's test. The experimental model for immunological indices was (Equation 1);

$$Y_{ijk} = \mu + A_i + B_j + (AB)_{ij} + \varepsilon_{ijk} \quad (1)$$

Where,
Y_{ijk} = Dependent variables
μ = Population mean
A_i = Effect due to ith fixed dose of vitamin C
B_j = Effect due to jth exposure time during acute heat stress (j = 0, 30, 60)
$(AB)_{ij}$ = Interaction between fixed dose of vitamin C and exposure time during acute heat stress
ε_{ijk} = Residual error

Results

Effect of fixed dose of vitamin C administration

The effects of fixed dose of vitamin C on the immunological cytokines and total haemocytes counts of Giant African Land snail (*Archachatina marginata*) during acute heat stress is shown in Table 2. The interferon gamma (IFN-γ) levels of snails in groups nAHS+VC or nAHS+nVC, were not significantly ($p > 0.05$) different

[1] Note: Standards concentration followed for interferon gamma and interleukin 2 were 800, 400, 200, 100, 50, 0 pg mL^{-1} and 8, 4, 2, 1, 0.5, 0 ng mL^{-1} respectively.

from each other. While snails in AHS+VC recorded elevated significant ($p < 0.05$) values compared to the group AHS+nVC. For interleukin 2 (IL-2), there was no significant ($p > 0.05$) difference across the treatment groups. However, total haemocyte counts (THC) in group of snails in nAHS+VC or nAHS+nVC had lower significant ($p < 0.05$) values compared to those in AHS+VC or AHS+nVC The total haemocyte counts increased under acute heat stress, and reduced with vitamin C administration.

Table 2. Effect of fixed dose of vitamin C on immunological cytokines and total haemocytes count of Giant African Land snail (*Archachatina marginata*) during acute heat stress (mean ± SE).

Parameter	Treatment			
	nAHS+nVC	nAHS+VC	AHS+nVC	AHS+VC
IFN-γ (pg mL^{-1})	7.80 ± 1.360^{b}	7.20 ± 1.580^{b}	73.20 ± 32.230^{b}	606.33 ± 302.860^{a}
IL-2 (ng mL^{-1})	0.019 ± 0.003	0.021 ± 0.001	0.029 ± 0.007	0.018 ± 0.005
THC (x 10^{6} cells mL^{-1})	18.25 ± 7.910^{b}	28.50 ± 7.910^{b}	89.92 ± 7.910^{a}	69.58 ± 7.910^{a}

^{a,b} Means with different superscripts in the same row differ significantly ($p < 0.05$). IFN-γ = Interferon gamma, IL-2 =Interleukin 2, THC = Total haemocyte count.

Effect of acute heat stress exposure time

The effects of exposure time on the immunological cytokines and total haemocytes counts of Giant African Land snail (*Archachatina marginata*) during acute heat stress is shown in Table 3. At 30 and 60 minutes, higher significant ($p < 0.05$) IFN-γ values were recorded compared to that at 0 minute. There was no significant ($p > 0.05$) difference at the exposed time for interleukin 2 (IL-2). However, at 0 min the THC had the least value which significantly ($p < 0.05$) differ at 30 minutes, and also was significantly ($p < 0.05$) different from those at 60 min. with highest THC (25.25 ± 6.85 x 10^{6} vs. 44.63 ± 6.85 vs. 83.31 ± 6.85 cells mL^{-1}) respectively.

Table 3. Effect of acute heat stress exposure time on immunological cytokines and total haemocytes counts of Giant African Land snail (*Archachatina marginata*) during acute heat stress (mean ± SE).

Parameter	Exposure Time		
	0 min.	30 min.	60 min.
IFN-γ (pg mL^{-1})	8.450 ± 2.436^{b}	286.050 ± 189.947^{a}	226.400 ± 151.333^{a}
IL-2 (ng mL^{-1})	0.020 ± 0.003	0.025 ± 0.006	0.021 ± 0.003
THC (x 10^{6} cells mL^{-1})	25.250 ± 6.850^{c}	44.630 ± 6.850^{b}	83.310 ± 6.850^{a}

^{a,b,c} Means with different superscripts in the same row differ significantly ($p < 0.05$). IFN-γ = Interferon gamma, IL-2 =Interleukin 2, THC = Total haemocyte count.

Interaction effect of fixed dose of vitamin C administration and acute heat stress exposure time

The interaction effects of fixed dose of vitamin C and exposure time on immunological cytokines and total haemocytes counts of GALS during acute heat stress are shown in Figures 1-3. In Figure 1, snails with vitamin C under acute heat stress at 30 minutes recorded significantly ($p < 0.05$) higher values for IFN-γ than at 0 minute with least value. At 60 minutes, there was no significant ($p > 0.05$) difference from 30 minutes (3.2 ± 0.86 vs. 1108 ± 678.328 vs. 707.8 ± 588.699 pg mL^{-1}).

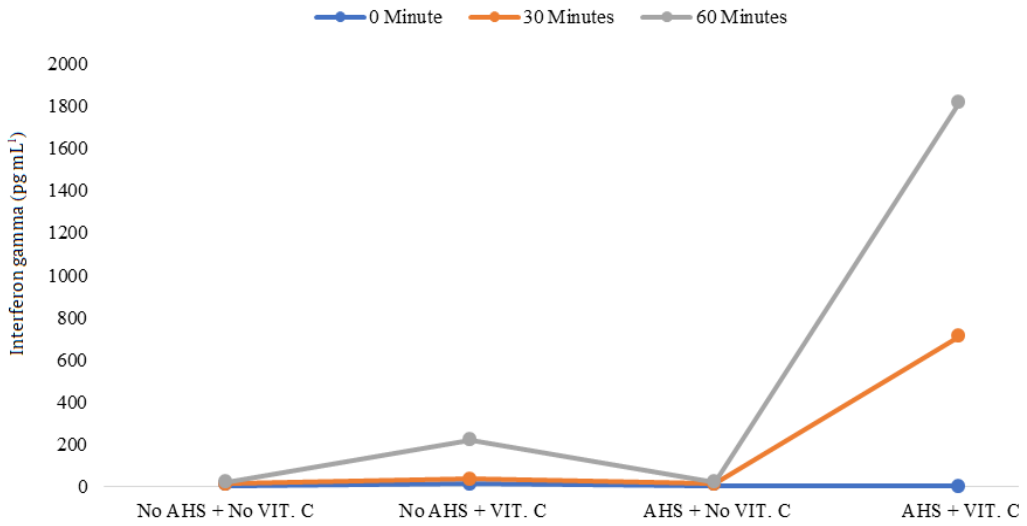

Figure 1. Interaction effect of fixed dose of vitamin C and exposure time on IFN- γ (pg mL^{-1}) concentration of Giant African Land snail (*Archachatina marginata*) under acute heat stress.

In Figure 2, it is clear from the study that snails without acute heat stress (with or without vitamin C) recorded stable values for IL-2. The group of snails with or without vitamin C during acute heat stress had no significant ($p > 0.05$) difference for IL-2 at all exposure times.

As presented in Figure 3, total haemocyte count (THC) of groups that did not experience acute heat stress with or without vitamin C had even values for THC at the all exposure times. While snails administered with or without vitamin C under acute heat stress recorded significant ($p < 0.05$) highest values at 60 minutes and least at 0 minute.

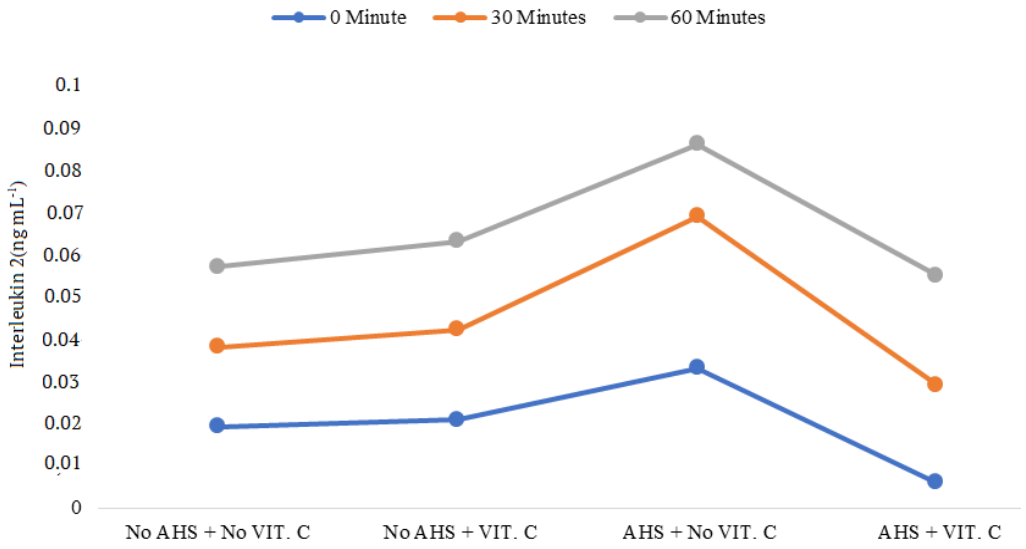

Figure 2. Interaction effect of fixed dose of vitamin C and exposure time on IL-2 (ng mL^{-1}) concentration of Giant African Land snail (*Archachatina marginata*) under acute heat stress.

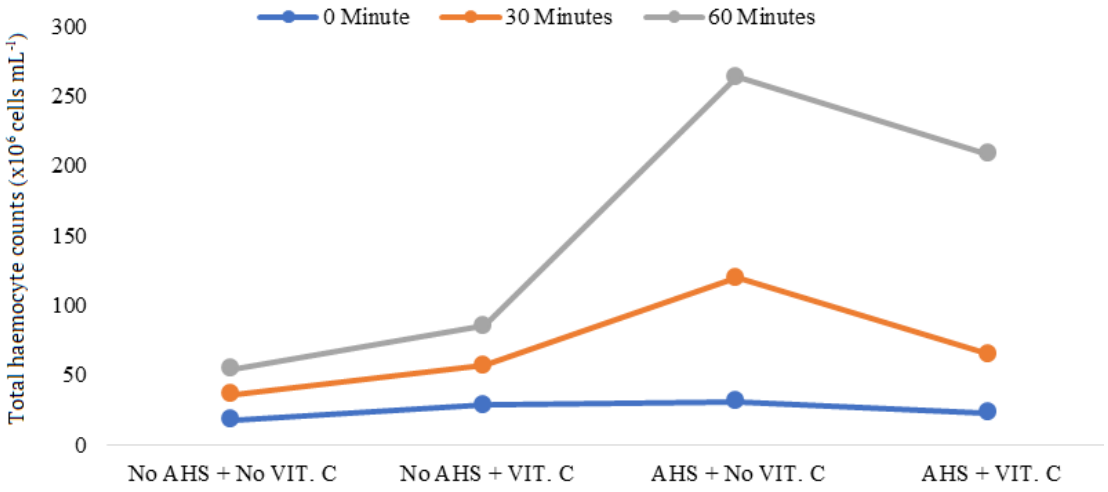

Figure 3. Interaction effect of fixed dose of vitamin C and exposure time on THC (x 10^6 cells/ml) concentration of Giant African Land snail (*Archachatina marginata*) under acute heat stress.

Discussion

The interferon gamma (IFN-γ) elevation in the study may be attributed to the effective utilization of the vitamin C in the maintenance of the immune state of land snails to heat stress. The finding is supported in the study of Jang, Ko, Moon, & Sohn, (2014) who observed that vitamin C administration to birds played significant role in regulation of IFN-γ. The surge in IFN-γ may be in activation of the haemocytes to exhibit immunoregulatory and immunomodulatory activities to eliminate resultant effect of the acute heat stress to land snails. Likewise, IFN-γ share more important biological activities in organisms thus, the major regulators of the host defense processes and as such are involved in the phagocytosis of foreign agent (Beschina, Bilej, Torreele, & Baetselier, 2001; Saito, 2001). The interleukin 2 (IL- 2) was not significantly affected by the vitamin C administration under acute heats stress in the present study. Marcus et al. (1991) contrarily,

observed that vitamin C was needed in the function of the immune system, particularly in the mediation of cells for immunity, which is usually triggered and responded with IL-2 in maintenance of immune status. The elevated total haemocyte counts under heat stress upon administration of vitamin C may be connected to the release of endocrine molecules in as response to impending dangers attributed to the stress condition. Similarly, in the study of Ottaviani, Franchini, and Fontanili (1992), it was reported that haemocytes are significantly involved in the stress response by secreting vertebrate like molecules in defense against foreign agents. The lower total haemocyte counts with the administration of vitamin C under no AHS, implies that the Giant African Land snail (GALs) had less challenge in fighting against foreign bodies usually triggered under AHS. Kambale and Potdar (2010) reported haemocytes as the cellular component in the haemolymph of Giant African Land snail which pose utmost defense mechanisms against foreign material. But vitamin C are known to boost the immune functions by the mediation of the cells (Marcus et al., 1991) increasing the total haemocytes counts.

The elevated IFN-γ values as the exposure times increased suggest the immunoregulatory role of the haemocytes against the acute heat stress. Accordingly, Ottaviani, Malagoli, & Franchini (2003) support that cytokines response to external environmental deviation to the animal immunological system.The IL-2 were not affected by the exposure time in this study. Loker (2010) stated that haemocytes of haemolymph are dependent on numerous factors like environmental variations for its activities. Thus, the increased total haemocyte counts in this study signify the responsive effects of the snails to adequate immune intervention. Further increase, may lead to exhaustion of the cells and hence death of the animals. So, it is known that haemocytes play key roles in internal defenses of land snails (Cheng, 1981).

The IFN-γ was at its best to ensure adequate immune response to the stress with the administration of vitamin C in combating heat stress. The observation of the improved IFN-γ concentration to boost immunity in this study, is in accordance with the study of Ottaviani et al. (2003) who reported that IFN -γ moderate the equilibrium of acquired and cellular immune responses. Yun, Moon, Sohn, and Jang (2012) found out that vitamin C supplementation during heat stress in rodents suppressed cytokines level. High and suppressed values of IL-2 in the interaction of vitamin c administration and exposure time could be attributed to the antioxidant property of the vitamin C in mitigating the inflammatory response of cytokines. Amarakoon, Tappia, and Grimble (1995) reported pro-inflammatory response of the cytokines expression in rodents. The large population of the haemocytes sustained could be attributed to the exposure of the GALs to the extreme heats challenge, which enabled the snails to measure up for internal defense against the stress condition. Cheng (1981) also reported active role of total haemocytes counts in internal defense of land snails.

Conclusion

With the increasing challenge of global warming on the ectotherms in the tropics, utilization of immune stimulating supplements to enhance the immune state of the animal during adverse environmental conditions are very key. It could be concluded in this study that fixed dose of vitamin C significantly elevated IFN-γ values during acute heat stress period better compared to snails without vitamin C administration providing improved effects to the animals. While the fixed dose of vitamin C had no significant effect on IL-2 released during the acute heat stress period. However, AHS increased total haemocyte counts upon vitamin C administration compared to the under no AHS condition. Interferon gamma and total haemocyte count levels increased with exposure time. It is therefore recommended that further research be carried out on immunomodulatory effect of fixed dose of vitamin C at 150 mg kg^{-1} of feed during acute heat stress.

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