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Alterations of biochemical indicators in hepatopancreas of the golden apple snail, *Pomacea canaliculata*, from paddy fields in Taiwan

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Abstract

The freshwater golden apple snail, *Pomacea canaliculata*, is one of the world's 100 worst invasive alien species. The snails' wide distribution, high abundance, and sensitivity to environmental pollution make them a potential bioindicator for environmental contamination. In this study, the biochemical status of golden apple snails collected from paddy fields throughout the island of Taiwan was examined. This study found that the biochemical status of apple snails collected from paddy fields differed from that of animals bred and maintained in the laboratory. Furthermore, certain biochemical endpoints of the snails collected from the paddy fields before and after agricultural activities were also different–hemolymphatic vitellogenin protein was induced in male snail after exposure to estrogen-like chemicals, the hepatic monooxygenase (1.97±0.50 $\triangle A_{650mm}$ 30 min^{-1} mg^{-1} protein in control group) and glutathione S transferase (0.02±0.01 $\triangle A_{340mm}$ 30 min^{-1} mg^{-1} protein in control group) snails exposed to pesticides, as well as the hepatopancreatic levels of aspartate aminotransferase (450.00±59.40 U mg^{-1} mg^{-1} protein in control group) and alanine aminotransferase (233.27±42.09 U mg^{-1} mg^{-1} protein in control group) decreased the indicating that xenobiotics destroyed hepatopancreatic. The above findings reveal that apple snail could be used as a practical bioindicator to monitor anthropogenic environmental pollution.

Key words

Agricultural activities, Biochemical status, Bioindicator, Golden apple snail

Introduction

An alien species is an organism that arrives in or is inadvertently introduced to a new geographic location or habitat by humans for some specific purpose, such as cultivation of food. If an alien species acclimatizes and successfully reproduces within the same niche in a new habitat previously occupied by native species, it is generally termed an invasive species. The negative effects of invasive species on native species often cause ecological, environmental and economic problems. The freshwater golden apple snail, *Pomacea canaliculata*, is the native of Amazon Basin of South America and has spread widely to USA (Rawlings *et al.,* 2007) and many Asian countries (Joshi, 2007) in recent decades, becoming one of the world's 100 worst invasive alien species (GISD, 2012). Golden apple snails were initially introduced to Taiwan in 1979 for cultivation as food. Although the snail is a low-calorie and potentially healthy food, the industry for its cultivation soon failed because of the snail's poor texture and high processing costs, and large numbers of golden apple snails were discarded by farmers and released into the country's fields. The golden apple snail is a voracious nocturnal herbivore and can destroy newly transplanted or direct-seeded rice by cutting the base of the seedling with its radula and chewing on the sheaths of rice, leading to significant loss to the yield (Joshi, 2007). The influence of golden apple snail on human health is also a concern because it is a host to parasite *Angiostrongylus cantonensis*, which causes eosinophilic meningoencephalitis (Mochida, 1991). Furthermore, the use of certain molluscicides by rice farmers against the golden apple snails leads to substantial impact on the environment because most of these chemicals are persistent and toxic/lethal to non-target, beneficial aquatic organisms (Joshi, 2007). The invasion of golden apple snails on Philippine agriculture was estimated to be between USD $ 425 and USD $ 1200 million in 1990 (Naylor, 1996).

The idea of using aquatic organisms as biological indicators to monitor environmental contamination has often been proposed. Conventionally, naturally occurring endemic species are the organisms of choice (Knakievicz and Ferreira, 2008). After their introduction to Taiwan more than thirty years ago, golden apple snails have become widely distributed in many different freshwater areas, indicating that they are well adapted to local climate and environment. In addition, golden apple snails were found to be sensitive to several environmental pollutants, such as metals and pesticides (Pena and Pocsidio, 2007; Ruamthum et al., 2010). Furthermore, their troublesome abundance in rice fields might be an additional advantage for environmental monitoring, as the "samples" will not be exhausted. Currently, alterations in the gonadal structure (imposex) of golden apple snails are used to reflect the presence of organotin compounds in the fields of Taiwan (Liu *et al.,* 2006; Wu *et al.,* 2010), suggesting that, despite their status as a notoriously invasive species, golden apple snails could be utilized as potential bioindicators for environmental contamination.

Although the practice of organic farming is increasing in developed countries, most of the agriculture in Taiwan is still based on traditional methods that rely heavily on chemical pesticides. However, the ecological impacts of the application of pesticides on paddy fields are not well known. Therefore, the objective of this study was to investigate the biochemical status of golden apple snails in paddy fields before and after harvest throughout Taiwan. These include hemolymphatic vitellogenin (VTG), the activities of hepatic monooxygenase (MO), glutathione *S* transferase (GST), aspartate aminotransferase (AST), and alanine aminotransferase (ALT), and the levels of hepatic creatinine (CREA) and urea.

Materials and Methods

Study sites and collection of animals : Studies were conducted in four paddy fields throughout Taiwan. The four paddy fields surveyed were Hsinchu (Hc, N24°92'09.2", E121°04'42.9"), Yunlin (Yl, N23°72'04.9", E120°42'85.6"), Tainan (Tn, N23°04'60.3", E120°20'70.3") and Pintung (Pt, N21°98'00.73", E120°73'08.47"), respectively. (Fig. 1). At each study site, animals were collected two weeks before transplantation of the rice seedlings and two weeks after the rice was harvested in 2011. At each sampling, 30-50 apple snails (~2-2.5 cm shell width) were collected, followed by immediate transport to our laboratory to determine the content of hemolymphatic VTG, the activities of hepatic drug-metabolizing enzymes, including MO and GST, and the levels of hepatic biochemical parameters, including AST, ALT, CREA and urea. In this study, golden apple snails of same size as the wild-caught animals that were maintained and bred in our laboratory for more than one generation were used as control group. These snails were reared in tanks equipped with a water-cycling device and maintained at the following water-quality values: a pH of 7.4-7.8; dissolved oxygen (DO) greater than 7.3 mg l^{-1}; hardness of 38-45 mg as $CaCO_3$ l^{-1}; and a temperature of 25 ± 1 °C under 12-h light-dark photoperiod regime.

Estimation of VTG : For estimating hemolymphatic VTG in male snail, the hemolymph of golden apple snails was extracted and stored at -20 °C until analysis. The hemolymphatic VTG protein content in the golden apple snail was determined using an alkali-labile phosphate (ALP) measurement assay, as previously described (Huang et al., 2006). The mixture of the supernatant (50 µl) and ice-cold 20% trichloroacetic acid (50 µl) was incubated at

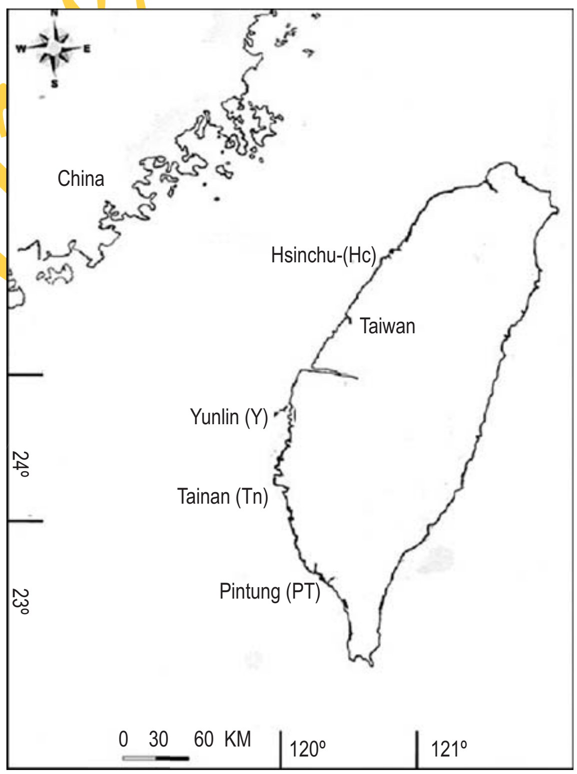

Fig. 1 : Locations of sampling apple snail collected from rice field in Taiwan

room temperature for 15 min, followed by centrifugation at 10,000 x *g* for 10 min at 4 °C. The protein pellet was resuspended in 200 µl 1 M NaOH and then heat-treated at 75 °C for 60 min before analysis. The level of free phosphate was determined by phospho molybdenum method (Stanton, 1968), and the optical absorbance at 600 nm was recorded with a microplate reader (PowerWare 340, BioTek, USA).

Determinations of drug-metabolizing and biochemical enzymes : The hepatopancreas was dissected, weighed and homogenized in ice-cold 25 mM Tris-HCl buffer containing 0.5 M EDTA (pH 8). The homogenate was then centrifuged at 10,000 x *g* for 40 min at 4 °C, and the supernatant was stored at -20 °C until analysis. The MO and GST activities were determined using reported methods (Brogdon and Barber, 1990; Brogdon *et al.,* 1997; Hemingway, 1998). The AST and ALT activities and CREA and urea levels in the hepatopancreas were determined using commercial kits (Cat NO. AL1268, Cat NO. AS1267, Cat NO. UR 221, and Cat NO. CR 510, respectively, RANDOX). The total protein content was determined using a commercial protein determination kit (Cat NO. 610-A, Sigma).

Statistical analysis : Statistical analysis was performed using SPSS ver. 12.0 (SPSS, 2003) software. One-way analysis of variance (ANOVA) was used to determine the effects of agricultural practices on the drug-metabolizing enzyme activities and biochemical parameters in the wild-caught apple snails. A *post hoc* Duncan's multiple-range test was used to evaluate the mean differences between control and wild-caught groups. The data for VTG did not meet the assumptions of normality, and, thus, were analyzed with a Mann-Whitney U test. A value of $P < 0.05$ was accepted as statistically significant.

Results and Discussion

Hemolymphatic VTG protein was detected in approximately 60% of the male golden apple snails collected from paddy fields (Table 1). At different study sites, the percentage of animals in which VTG protein was detected ranged from 0 to 100%, with the level ranging from 0 to 414.32 µg g^{-1}. The percentage of animals in which VTG protein was detected before transplantation of the rice seedlings differed significantly from that after the rice was harvested at Pt but not at Hc, Yl and Tn. The hemolymphatic VTG protein was not detected in control snails.

MO activity in the hepatopancreas of control apple snails was 1.97 $\triangle A_{650mm}$ 30 min^{-1} mg^{-1} protein (Fig. 2A). A significant elevation in the hepatopancreatic MO activity was observed in the wild-caught snails from Hc, Tn and Pt before transplantation of the rice seedlings ($p < 0.05$). Similarly, the hepatopancreatic MO activities of the golden apple snails collected in Hc, Yl, and Pt after the rice was harvested were significantly higher than those of the control ($p<0.05$). A significant difference in the hepatopancreatic MO activities of the wild-caught snails before transplanting and after harvest was also observed for Hc, in that the MO activity in the animals collected after rice harvest was higher ($p < 0.05$), and Tn, in that the MO activity in the animals collected before the rice transplantation was higher ($p < 0.05$).

The hepatopancreatic GST activity in control snails was 0.02 $\triangle A_{340mm}$ 30 min^{-1} mg^{-1} protein (Fig. 2B). The hepatopancreatic GST activities of the snails collected before rice transplantation were significantly higher than control ($p < 0.05$). Also, the hepatopancreatic GST activities in animals collected from Hc, Yl and Pt after harvest were higher than control ($p < 0.05$). The hepatopancreatic GST activities of the wild-caught snails before transplanting and after harvest were different for Yl and Tn, in that the GST activities in the animals collected before transplantation of the rice seedlings was higher ($p < 0.05$), and Pt, in that the activity in the animals collected after rice harvest was higher ($p < 0.05$).

The hepatopancreatic ALT and AST activities of the control golden apple snails were 233.27 and 450.00 U mg^{-1} mg^{-1} protein, respectively (Fig. 3A, B). The hepatopancreatic ALT activities in the snails collected in Hc, Tn, and Pt before transplantation of rice seedlings and in Hc, Yl, and Pt after rice was harvested were significantly lower than control ($p < 0.05$). However, the hepatopancreatic ALT activity in the snails collected in Yl before transplantation of rice seedlings was higher than control ($p < 0.05$). Significant differences before transplanting and after harvest were observed for Hc, Yl and Pt. Interestingly, the pattern of hepatopancreatic AST activity in the wild-caught golden apple snails was almost comparable to that of ALT activity, and

Table 1 : Level of VTG protein and incidence rate (%) in male golden apple snail collected from rice field before (1^{st}) and after (2^{nd}) harvested in Taiwan

	Control	Study site							
		Hsinchu		Yunlin		Tainan		Pingtung	
		1^{st}	2^{nd}	1^{st}	2^{nd}	1^{st}	2^{nd}	1^{st}	2^{nd}
Incidence rate (%) (number of snails for which VTG was detectable/number of snails examined)	0 (0/5)	33 (1/6)	100 (20/20)	27 (5/15)	94 (16/17)	69 (9/13)	59 (10/17)	0 (2/4)	0^a (0/20)
Average level of VTG (ig/g TP)	ND	78.49	78.77	11.3	289.26*	35.39	414.32*	1.08	ND

ND: results under the detection limit. *the results from the 1^{st} and 2^{nd} samplings were significantly different ($p < 0.05$) using the Mann-Whitney U test significant differences before transplanting and after harvest were observed for Hc, Yl and Tn.

In control snails, the concentration of hepatopancreatic CREA was 1.20 $\mu g\ mg^{-1}$ protein (Fig. 3C). The hepatopancreatic concentrations of CREA in the wild-caught snails were significantly higher than control, except for those collected in Hc before transplantation of rice seedlings and Pt after rice was harvested. Significant differences before transplanting and after harvest were observed for Hc and Pt.

The urea concentration in hepatopancreas of control snails was 30.00 $\mu g\ mg^{-1}$ protein (Fig. 3D). The hepatopancreatic urea concentration in golden apple snails collected in Tn before transplantation of rice seedlings was significantly lower than control animals. However, the hepatopancreatic urea concentration was significantly higher than control for the snails collected in Pt after rice was harvested. Significant differences before transplanting and after harvest were observed for Tn and Pt.

Information on the toxic responses of golden apple snails to environmental chemicals was limited to their acute sensitivity to toxins (Lo and Hsieh, 2000), bioaccumulation patterns (Deng et al., 2008; Fu et al., 2011; Vega et al., 2012), histopathology (Kruatrachue *et al.,* 2011), feeding rates, growth and reproduction (Pena and Pocsidio, 2007). Although the biochemical responses of golden apple snails to certain xenobiotics that were considered as having potential molluscicidal activity were investigated for the development of molluscicides (Dai *et al.,* 2011; Ruamthum *et al.*, 2010), the responses of the snails to environmental contaminants were rarely investigated.

Pesticides are the major chemicals used by farmers during traditional agricultural activities in Taiwan. Due to serious impact on agriculture caused by golden apple snails, molluscicides—such as triphenyltin and tributyltin, were one of the principal pesticides used by farmers. The levels of triphenyltin and tributyltin residues in sediment samples from paddy fields was directly estimated (TACTRI, 2003) and indirectly by observing of the incidence of imposex in golden apple snails as a biomarker (Liu et al., 2006).

To develop golden apple snails as a potential bioindicator for agricultural activities in fields, it is critical to select the biochemical parameters that can adequately reflect the use of chemical pesticides by farmers and the existence of residues in the fields as specifically as possible. It has been report that the abnormal expression of VTG in the animals was a practical biomarker for xenoestrogen exposure (Huang *et al.,* 2006; Rodas-Orti'z *et al.*, 2008; Blaise *et al.*, 1999). Additionally, cytochrome P450 (CYP) and GST are key enzymes involved in phase I and II detoxification mechanisms, respectively, and their expression levels and activities were significantly altered by many xenobiotics (Bouraoui *et al.,* 2008; Ezemonye and Tongo, 2010; Tierney *et al.,* 2008). MO activity to represent the content of CYP enzymes reflects the heme content of non-bloodfed invertebrates, the majority of which was associated with CYP (Brogdon *et al.,* 1997). Increases in hepatic MO activity were observed in animals treated with organic chemicals (Marsili *et al.,* 2009; Stouvenakers *et al.,* 1996; Wang *et al.,* 2001). Similarly, elevated hepatic GST activity was found in pesticide-treated rainbow trout, *Oncorhynchus mykiss* (Tierney *et al.,* 2008). In this study VTG protein was detected in most of the animals collected from study sites. Clearly, this observation implied that these wild-

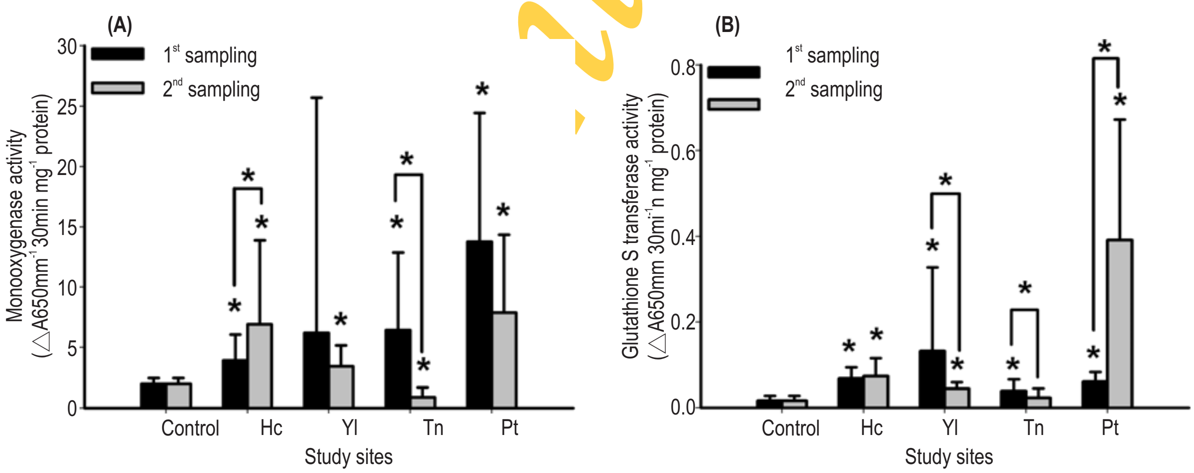

Fig. 2 : (A) Monooxygenase (MO) and (B) glutathione S transferase (GST) activities in the hepatopancreas of golden apple snails (N = 30-50) collected in the paddy fields of Taiwan. The control group (N = 5) included animals maintained and bred in our laboratory. The apple snail collected from rice field before and after harvested showed 1st and 2nd sampling, respectively. *$p < 0.05$, compared to the corresponding control or between treatments

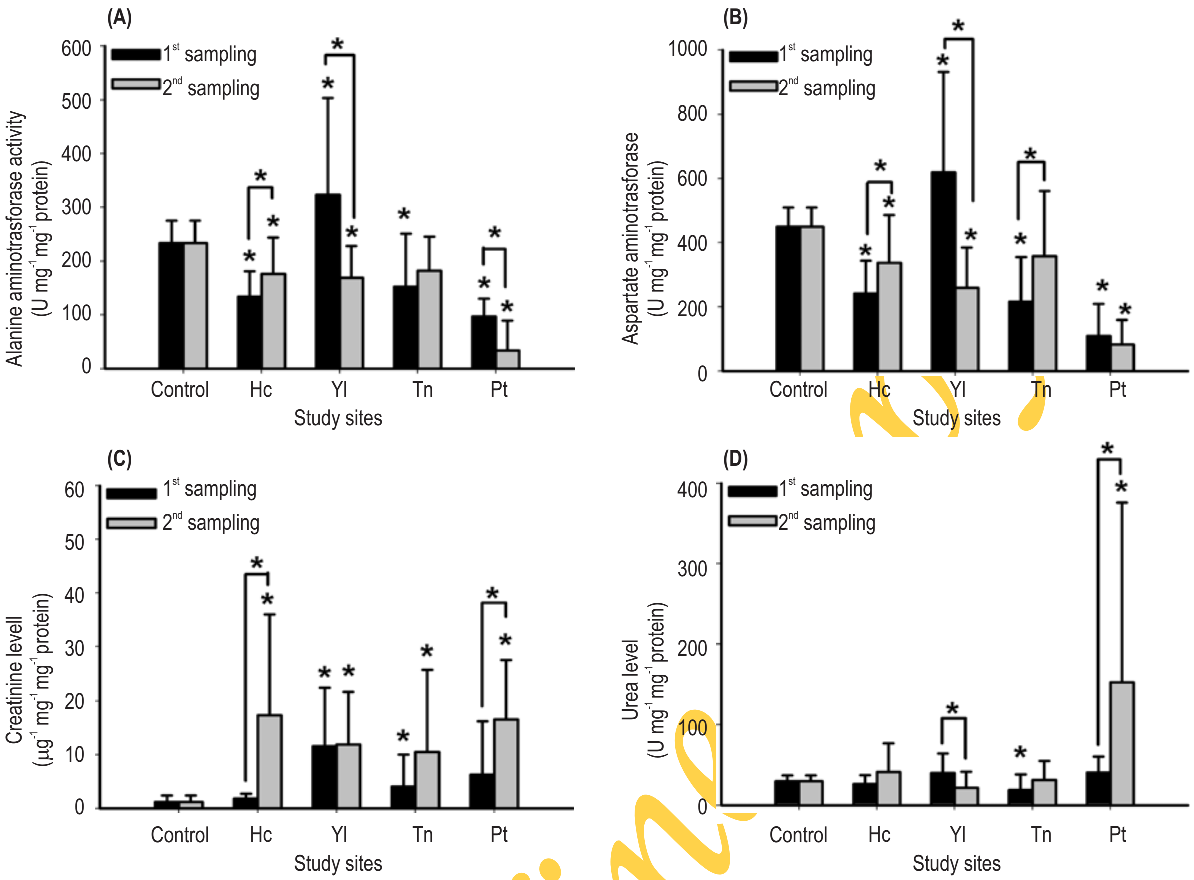

Fig. 3 : (A) Alanine aminotransferase (ALT) and (B) aspartate aminotransferase (AST) activities and (C) creatinine (CREA) and (D) urea levels in the hepatopancreas of golden apple snails (N = 30-50) collected in the paddy fields of Taiwan. The control group (N = 5) included animals maintained and bred in our laboratory. The apple snail collected from rice field before and after harvested showed 1st and 2nd sampling, respectively. *$p < 0.05$, compared to the corresponding control or between treatments

caught snails were exposed to xenoestrogen(s) that were distributed in the sediment and/or water of the paddy fields.

AST and ALT are important liver enzymes used as diagnostic tools for detecting the toxic effects of various pollutants (Klaassen, 2001). Increases in serum level and decreases in hepatic/hepatopancreatic level of AST and ALT were demonstrated in toxicant-treated aquatic animals and, thus, were used as practical indicators hepato-/hepatopancreatic toxicity of xenobiotics (Kim and Kang, 2004; Vaglio and Landriscina, 1999; Wu *et al.,* 2008). The hepatopancreatic level of AST and ALT in the golden apple snails collected from study sites showed comparable patterns, and most of these levels were significantly lower than those measured in control snails. Similarly, urea and CREA levels are clinical indicators of renal function. This result showed that, compared to control snails, the level of these indicators, especially CREA, in the snails collected from paddy fields were notably higher. These results indicate that the golden apple snails collected from paddy fields showed hepatopancreatic and renal toxicities, were most likely exposed to pesticide stressor(s), as pesticides had previously been correlated with hepatotoxic effect and renal failure (Betrosian *et al.*, 1995; Dahamna *et al.,* 2004; Ohno *et al.*, 1998; Wanigasuriya *et al.,* 2007). Based on the findings of VTG protein, MO, GST and biochemical parameter, it has been suggested that the apple snail could also be used as a practical bioindicator to monitor anthropogenic environmental pollution.

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