Research Article

International Journal of Agriculture and Wildlife Science

Uluslararası Tarım ve Yaban Hayatı Bilimleri Dergisi

2023, 9(3): 448 – 459, doi: 10.24180/ijaws.1315843

Investigation of Glutathione S-Transferase-Alpha and Glutathione S-Transferase-Pi Expression Levels in *Spermophilus xanthoprymnus* and *Meriones tristrami* in Terms of Living Conditions and Natural Habitat Differences in Kırıkkale Province

Kırıkkale İlinde *Spermophilus xanthoprymnus* ve *Meriones tristrami*'de Glutatyon S-Transferaz-Alfa ve Glutatyon S-Transferaz-Pi Ekspresyon Düzeylerinin Yaşam Koşulları ve Doğal Habitat Farklılıkları Açısından İncelenmesi

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Received: 17.06.2023 **Accepted:** 13.09.2023 **Published:** 20.12.2023

Abstract: Glutathione S-transferase (GST) is a multifunctional enzyme that provides homeostasis by catalyzing the first step in the formation of the end product mercapturic acid in the detoxification metabolic pathway. Being found in mammals, insects, fish, birds, annelids, molluscs, and many microorganisms, GST takes part the elimination of toxic substances taken into body by consuming food, and their transport by binding non-substrate ligands (e.g. heme and bilirubin) with GSH. In addition, it can prevent reactive electrophilic compounds from harming the body by covalent bonding similar compounds to each other. These xenobiotic acceptors affected by GST include nitrogen halogen compounds, organophosphates, and polycyclic aromatic hydrocarbons. Xenobiotics are oxygenated by this enzyme system, the next mechanism of oxygenated products is more oxygenation, and these products become more easily soluble in water. In this study, Glutathione S-Transferase was detected in the liver tissue of *Spermophilus xanthoprymnus* and *Meriones tristrami* and its characteristic features were determined. For this purpose, the animals were anesthetized with sodium pentobarbital and their liver tissues were harvested. After necessary preparations were completed, the samples were analyzed by using immunohistochemical staining method and the expressions of GST isozymes were determined. As a result, glutathione s-transferase-alpha and glutathione s-transferase-pi expression levels were found to differ in *Spermophilus xanthoprymnus* and *Meriones tristrami* samples obtained from different localities of Kırıkkale province. Differences in GST enzyme expression in these species indicate that both species differ in their detoxification capacity and response to xenobiotics.

Keywords: Glutathione S-transferase, habitat differences, immunohistochemistry, *Meriones tristrami*, *Spermophilus xanthoprymnus*

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Öz: Glutatyon S-transferaz (GST) çok fonksiyonlu bir enzim olup detoksifikasyon metabolik yolunda son ürün merkaptürik asit oluşumundaki ilk adımı katalize ederek homeostaz sağlar. Memeliler, böcekler, balıklar, kuşlar, annelidler, yumuşakçalar ve birçok mikroorganizmada bulunan GST, besinlerin tüketilmesiyle vücuda alınan toksik maddelerin vücuttan atılmasında ve substrat olmayan ligandlara (örn. heme ve bilirubin) bağlanarak taşınmasında görev alır. GSH. Ayrıca benzer bileşikleri birbirine kovalent yolla bağlayarak reaktif elektrofilik bileşiklerin vücuda zarar vermesini engelleyebilmektedir. GST'den etkilenen bu ksenobiyotik alıcılar, nitrojen halojen bileşikleri, organofosfatlar ve polisiklik aromatik hidrokarbonları içerir. Ksenobiyotikler bu enzim sistemi tarafından oksijenlenir, oksijenli ürünlerin bir sonraki mekanizması daha fazla oksijenlenme olur ve bu ürünler suda daha kolay çözünür hale gelir. Bu çalışmada *Spermophilus xanthoprymnus* ve *Meriones tristrami*'nin karaciğer dokusunda Glutatyon S-Transferaz saptanmış ve karakteristik özellikleri belirlenmiştir. Bu amaçla hayvanlara sodyum pentobarbital ile anestezi uygulandı ve karaciğer dokuları alındı. Gerekli hazırlıklar tamamlandıktan sonra örnekler immunohistokimyasal boyama yöntemi kullanılarak analiz edildi ve GST izozimlerinin ifadeleri belirlendi. Sonuç olarak, Kırıkkale ilinin farklı lokalitelerinden temin edilen *Spermophilus xanthoprymnus* ve *Meriones tristrami* örneklerinde glutatyon s-transferaz alfa ve glutatyon s-transferaz-pi ifade düzeylerinin farklı olduğu bulundu. Bu türlerdeki GST enzim ekspresyon farklılıkları her iki türün detoksifikasyon kapasitesinin ve ksenobiyotiklere vereceği cevabın farklı olduğunu gösterir.

Anahtar Kelimeler: Glutatyon S-transferaz, habitat farklılıkları, immünohistokimya, *Meriones tristrami, Spermophilus xanthoprymnus*

Cite as: Pamukoğlu, N., Oğuztüzün, S., Dirican, O. and Yılmaz Sarıaltın, S. (2023). Investigation of glutathione s-transferase-alpha and glutathione s-transferase-pi expression levels in spermophilus xanthoprymnus and meriones tristrami in terms of living conditions and natural habitat differences in Kırıkkale province. *International Journal of Agriculture and Wildlife Science*, 9(3), 448-459 doi: 10.24180/ijaws.1315843.

Plagiarism/Ethic: This article has been reviewed by at least two referees and it has been confirmed that it is plagiarism-free and complies with research and publication ethics. https://dergipark.org.tr/tr/pub/ijaws

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INTRODUCTION

Glutathione S-transferases (GSTs) are a member of the Phase-II detoxification enzyme family, which protects cellular macromolecules against reactive electrophiles by interacting electrophilic and hydrophilic compounds with glutathione (Ismail et al., 2021). Glutathione S-transferases are divided into three families: mitochondrial, cytosolic, and microsomal. GST isoenzymes are present in different amounts in various tissues (Ploemen et al., 1996). GSTs play an important role in the detoxification of electrophilic xenobiotics such as herbicides, pesticides, anticancer drugs, chemical carcinogens, and environmental pollutants. GST is found in a wide range of organisms ranging from E.coli to mammals and is examined from isolation from the liver, erythrocyte, lung, placenta and intestinal mucosa of living beings such as humans, rats, mice, and cattle (Moody et al., 1991). In an in vivo study investigating the effects of ten structurally diverse herbicides on xenobiotic metabolizing enzymes in mouse liver (Gyamfi et al., 2004), it was determined that when CDNB was used as a substrate on cytosolic GSTs, molinate, benthiocarb, trifluralin and alachlor caused a significant increase in activity. Since glutathione S-transferases have the ability to catalyze a large number of structurally diverse substrates such as lactones, alkyls, aryl halides, quinones, epoxides, and esters, comprehensive studies have been conducted on glutathione S-transferases.

In a study, the substrate properties of cytosolic glutathione S-transferase (GST) activity were compared in various marine fish, anadromous and freshwater salmonids. GST subunits were used to investigate phylogenetic relationships between purified rat polyclonal antiserum and mammalian and fish enzymes. GSTs were purified from various tissues of humans, mice, cattle and rats and their structures, functions and metabolic properties were extensively studied (Dominey et al., 1991). GSTs were first identified in rat liver, and then they were classified according to their substrate specificities. GSTs are classified under five groups as epoxide transferase, aryl transferase, alkyl transferase, alkene transferase, and aralkyl transferase. The enzymes are classified based on physical and structural properties of the protein instead of the enzymatic properties. GST subunits are divided into subclasses as alpha, mu, pi, theta, sigma, delta, beta, tau, and zeta forms (Mannervik et al., 2005). While alpha, pi and mu forms of GST play an important role in drug metabolism, the sigma subunit is significantly involved in prostaglandin synthesis (Lizuka et al., 1989). Zeta and theta forms are found in both plants and animals, while tau and pi forms are plant specific (Mannervik et al., 2005). It was investigated how Cd^{+2} and Mn^{+2} metal ions, which accumulate in nature and cause heavy metal poisoning in living beings, affect GST enzyme activity in rat liver. It was reported that when $CdCl_2$ (2.5 mg kg^{-1}) or $MnCl_2$ (2.0 mg kg^{-1}) amounts were administered as a single dose, enzyme activity increased by 36% after one day (Bocedi et al., 2019). Most of xenobiotics are highly hydrophobic and are located in hydrophobic parts of the cell that contain the membranes and side segment of certain soluble proteins. The endoplasmic reticulum in rat hepatocytes constitutes 50% of the cell membranes (Dixon et al., 2002). The reversible inhibition and time-dependent inactivation of the interaction of disulfiram (DSF), a drug used in the treatment of alcoholics, and diethyldithiocarbamate (DDTC) in its reduced form with rat and human liver GST, were investigated (Ismert et al., 2002). The human GST alpha 2–2 isoenzyme containing no cysteine had the greatest sensitivity. The results of that study showed that 1 thiol residue is involved in this inactivation and inactive GST can be reversed by administration of GSH or dithiothreitol. The effects of t-butyl hydroperoxide, cumene hydroperoxide and linoleic acid hydroperoxide on liver microsomal GST enzyme in rats were examined (Morgenstren et al., 1988). The data showed that microsomal GST was activated by more organic hydroperoxides in the presence or absence of GSH. The studies conducted with homogeneous enzyme preparations from rats and humans have revealed that they have binding proteins of similar size. These enzymes are adhesive and binding. The factors affecting the binding are associated with the hydrophobic regions of the ligand or whether the molecules are similar or different. The GST activity in male mice in a 24-hour period were examined (Llavanera et al., 2020). In human and rat GST enzymes, the most abundant amino acids are aspartic acid, glutamic acid, and leucine. Tryptophan and cysteine amino acids, on the other hand, are found in very low amounts in rats, but not at all in humans. The enzyme is thought to protect cells against foreign substances such as pesticides, drugs, and carcinogens.

To date, the properties of glutathione S-transferases, which have been very well purified from mammals such as humans, mice, cattle, and rabbits, have been studied in detail (Aniya and Daido, 1993).

The aim of this study was to reveal the metabolism differences between *Spermophilus xanthoprymnus* and *Meriones tristrami* and the detoxification enzyme levels in different tissues and organs of them due to toxic substance exposure in their habitats.

MATERIAL AND METHOD

Legal permissions were obtained from The Turkish Ministry of Agriculture and Forestry, General Directorate of Nature Conservation and National Parks (number: 2019/E.2467643) for the study. Four samples including ground squirrel (*Spermophilus xanthoprymnus*) and Tristram's jird (*Meriones tristrami*) were obtained from Kırıkkale province as a result of the field studies. In this study, the animals were dissected in the laboratory of Kırıkkale University.

Immunohistochemical Staining

The formalin-fixed tissue blocks were cut into 4µm sections and mounted onto poly-L-lysine-coated slides. For immunohistochemistry, sections, which were dewaxed in xylene and rehydrated in ethanol, were washed with distilled water for 3 minutes. Afterwards, the sections were peroxidase-incubated for 10 minutes using 3% hydrogen peroxide in methanol (v/v) and then they were washed with distilled water for 3 minutes. Antigen retrieval was performed for 3 minutes using a 0.01 M citrate buffer at pH 6.0 in a domestic pressure cooker.

The sections were placed in Tris-buffered saline (TBS) containing 0.15 M sodium chloride and 0.05 M Tris-HCL in pH 7.6. They were washed with water and then incubated at room temperature for 10 minutes with superblock (SHP125; Scy Tek laboratories, west logan, UT) to block non-specific background staining. The primary antibody was diluted through a diluting solution, based on the manufacturer company's instructions. The sections were incubated with the primary antibody for anti-GST alpha (bms-51742 M; Bioss Inc) diluted 1:200 and anti-GST pi (sc-66000; Santa Cruz Biotechnology.,Inc) diluted 1:200. After washing for 15 minutes in TBS, they were incubated at room temperature with a biotinylated link antibody (SHP125; ScyTek Laboratories) followed by streptavidin/HRP complex (SHP125; ScyTek laboratories). After washing with TBS for 15 min, the sections were incubated at room temperature with biotinylated link antibody (SHP125; ScyTek Laboratories). Then, diaminobenzidine was used to visualize peroxidase activity in tissues. Nuclei were lightly counterstained with hematoxylin and then the sections were dehydrated and mounted. Light microscopy and scoring of immunohistochemically stained sections were performed for each enzyme as: (-) negative (no staining); 1, weak staining; 2, moderate staining; and 3, strong staining.

Statistical Analysis

Data was analyzed by using SPSS 25.0 (IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp) software. Shapiro Wilk test was used to determine data distribution test. The Levene's Test was used for testing equality of variances. The Student t-Test was performed to compare normally-distributed groups. The Mann Whitney-U Test was performed to compare non-normally distributed groups. The Pearson's correlation analysis was used to examine the correlation between the data. A p-value less than 0.05 was accepted as statistically significant.

RESULTS

The cellular expression of two glutathione S-transferase-isozymes (GST pi, GST alpha) was examined in the liver, heart, small intestine, stomach, spleen, lung and kidney tissues of *Spermophilus xanthoprymnus* and *Meriones tristrami*. A wide variation was found in the cellular localization of these enzymes between these two species. There was negative staining for GST alpha in kidney tissue of *Spermophilus xanthoprymnus* but, strong staining for GST alpha in kidney tissue of *Meriones tristrami*. Moderate staining was detected for GST alpha in stomach tissue of *Spermophilus xanthoprymnus;* whereas, negative staining was observed in stomach tissue of *Meriones tristrami* (Figure 1 and Figure 2).

Moreover, while GST pi immunostaining was strong in kidney tissue of *Spermophilus xanthoprymnus*, it was negative in kidney tissue of *Meriones tristrami*. Additionally, while GST pi staining was negative in stomach and spleen tissues of *Meriones tristrami*, it was moderate staining was detected for the other species (Table 1).

A graphic showing the distribution of expressions of GST alpha and GST pi isozymes between *Spermophilus xanthoprymnus* (Species 1) and *Meriones tristrami* (Species 2) is given in Figure 3.

GST alpha expressions in tissues of species 1 and 2 was expressed in Figure 4. There was no statistically significant difference in GST alpha expressions between species 1 and 2 ($p=0.277$; $p>0.05$) (Figure 4).

GST pi expressions in tissues of species 1 and species 2 was shown in Figure 5. No statistically significant difference was observed in GST pi expressions between species 1 and 2 ($p=0.902$; $p>0.05$) (Figure 5).

Correlation analyses were performed to investigate the correlation between the staining intensities of GST alpha and GST pi. Figure 6 shows the expressions of GST alpha versus GST pi. A significant correlation was found between the staining intensities of GST alpha and GST pi. The Pearson's correlation coefficient (r) was 0.583 and the significance (2-tailed) was 0.028 based on observations ($p<0.05$) (Figure 6).

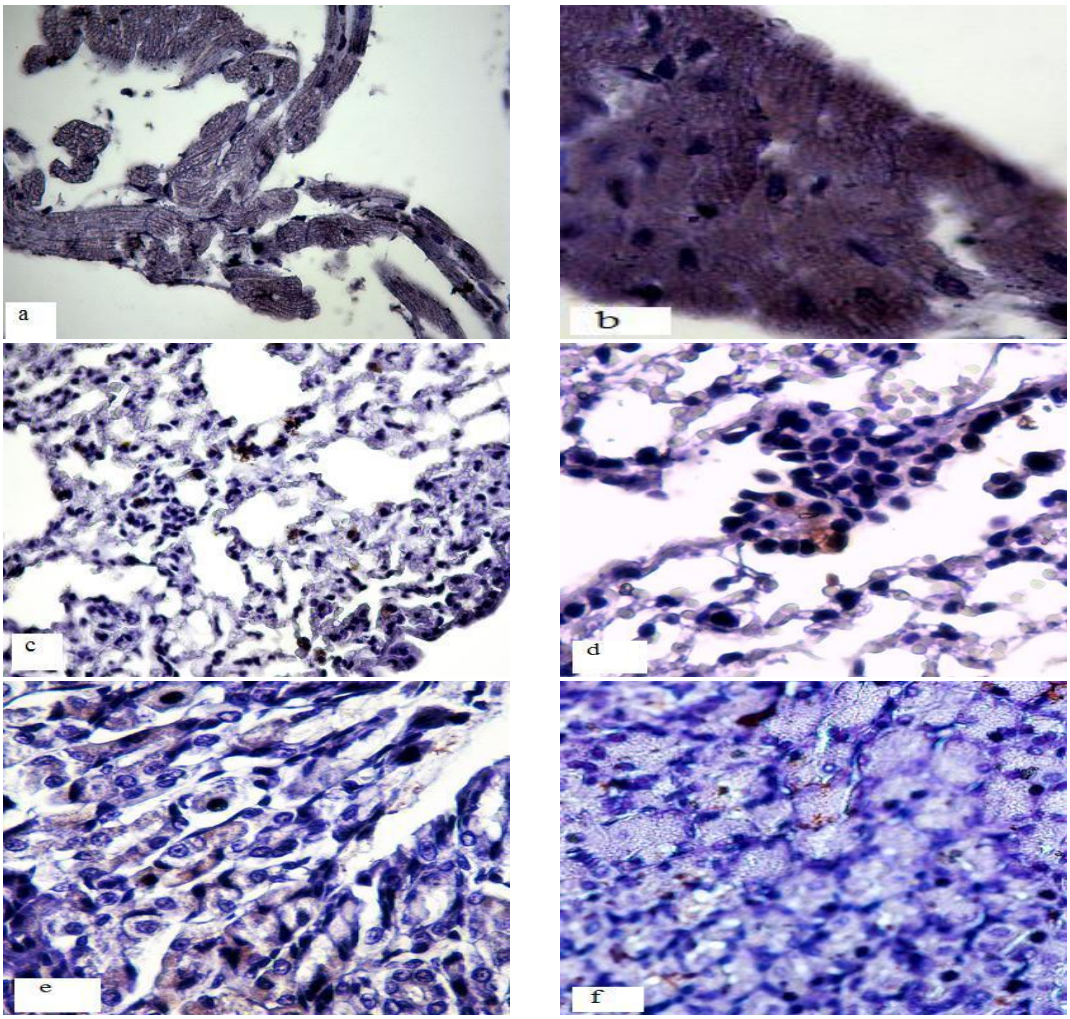

Figure 1. The cellular expression of two glutathione S-transferase alpha in tissues of *Spermophilus xanthoprymnus* (S) and *Meriones tristrami (M)* (a: heart S, b: heart M; c: lung S, d: lung M; e: stomach S, f: stomach M; g: liver S, h: liver M; i: spleen S, j: spleen M; k: kidney S, l: kidney M; m: small intestine S, n: small intestine M) (X400).

Şekil 1. Spermophilus xanthoprymnus (S) ve Meriones tristrami (M) dokularında iki glutatyon S-transferaz alfanın hücresel ifadesi (a: kalp S, b: kalp M; c: akciğer S, d: akciğer M; e: mide S, f: mide M; g: karaciğer S, h: karaciğer M; i: dalak S, j: dalak M; k: böbrek S, l: böbrek M; m: ince bağırsak S, n: ince bağırsak M) (X400).

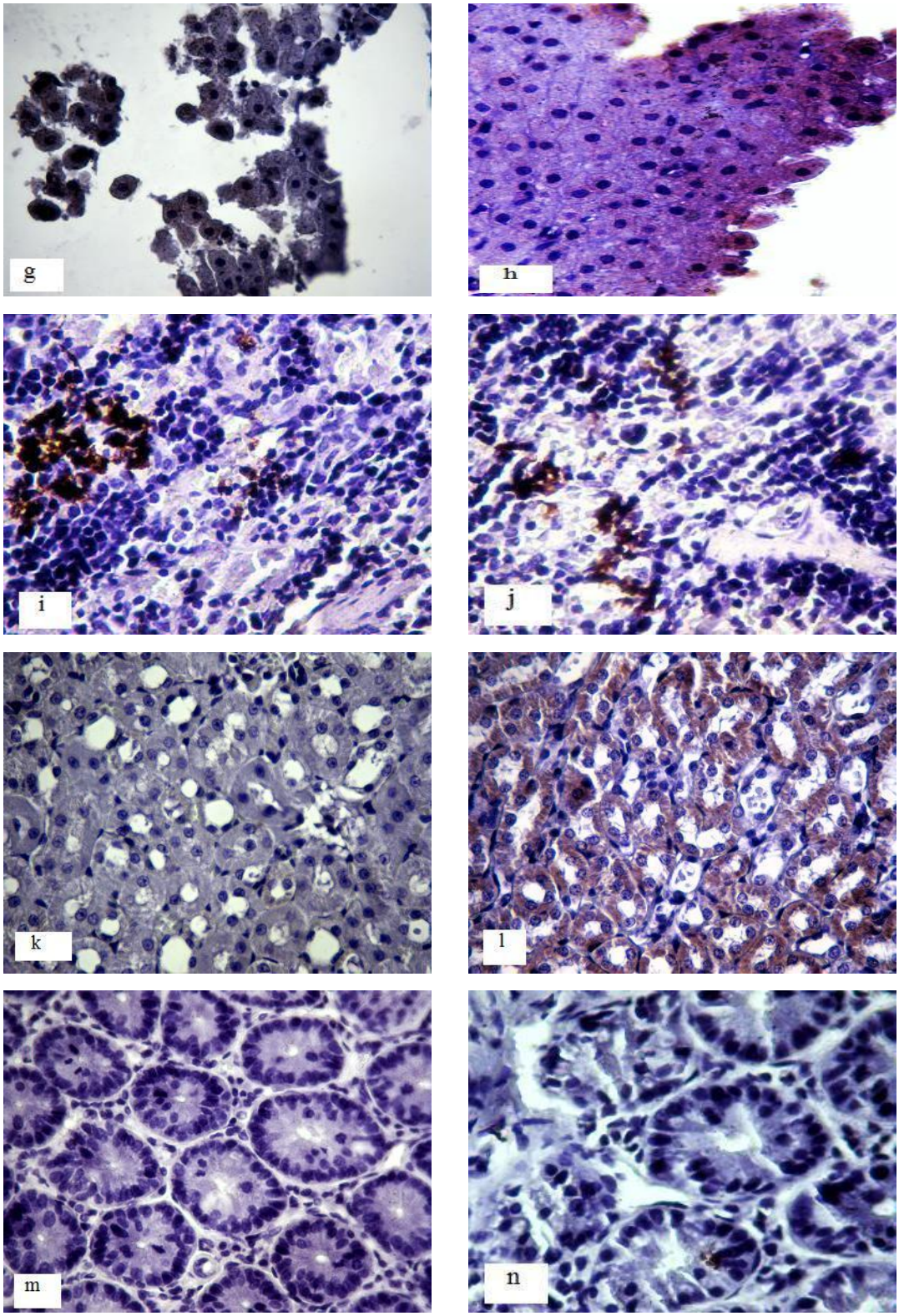

Figure 1. Continue.
Şekil 1. Devamı.

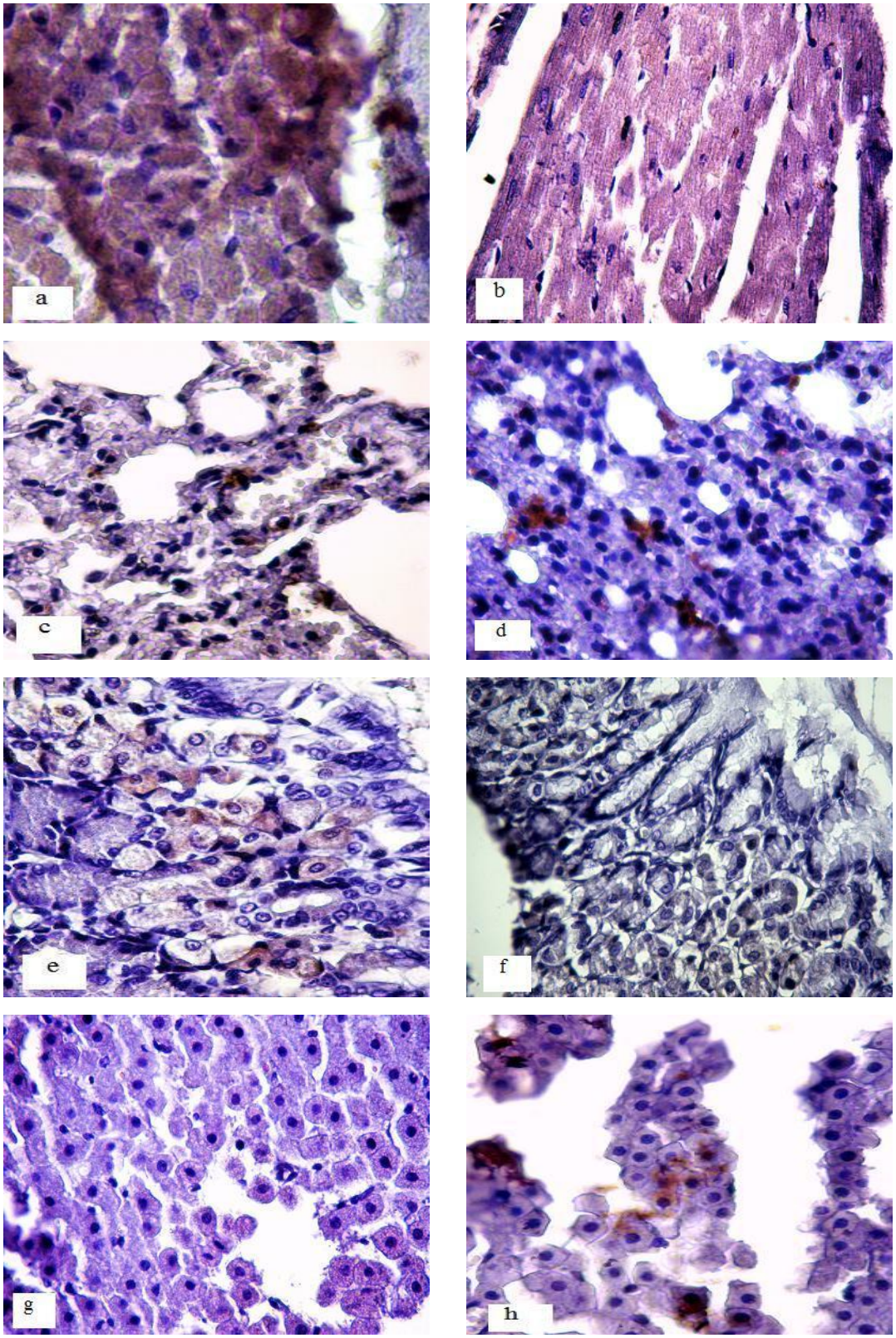

Figure 2. The cellular expression of glutathione S-transferase pi in tissues of *Spermophilus xanthoprymnus* (S) and *Meriones tristrami (M)* (a: heart S, b: heart M; c: lung S, d: lung M; e: stomach S, f: stomach M; g: liver S, h: liver M; i: spleen S, j: spleen M; k: kidney S, l: kidney M; m: small intestine S, n: small intestine M (X400).

Şekil 2. Spermophilus xanthoprymnus (S) ve Meriones tristrami (M) dokularında glutatyon S-transferaz pi'nin hücresel ifadesi (a: kalp S, b: kalp M; c: akciğer S, d: akciğer M; e: mide S, f : mide M; g: karaciğer S, h: karaciğer M; i: dalak S, j: dalak M; k: böbrek S, l: böbrek M; m: ince bağırsak S, n: ince bağırsak M (X400).

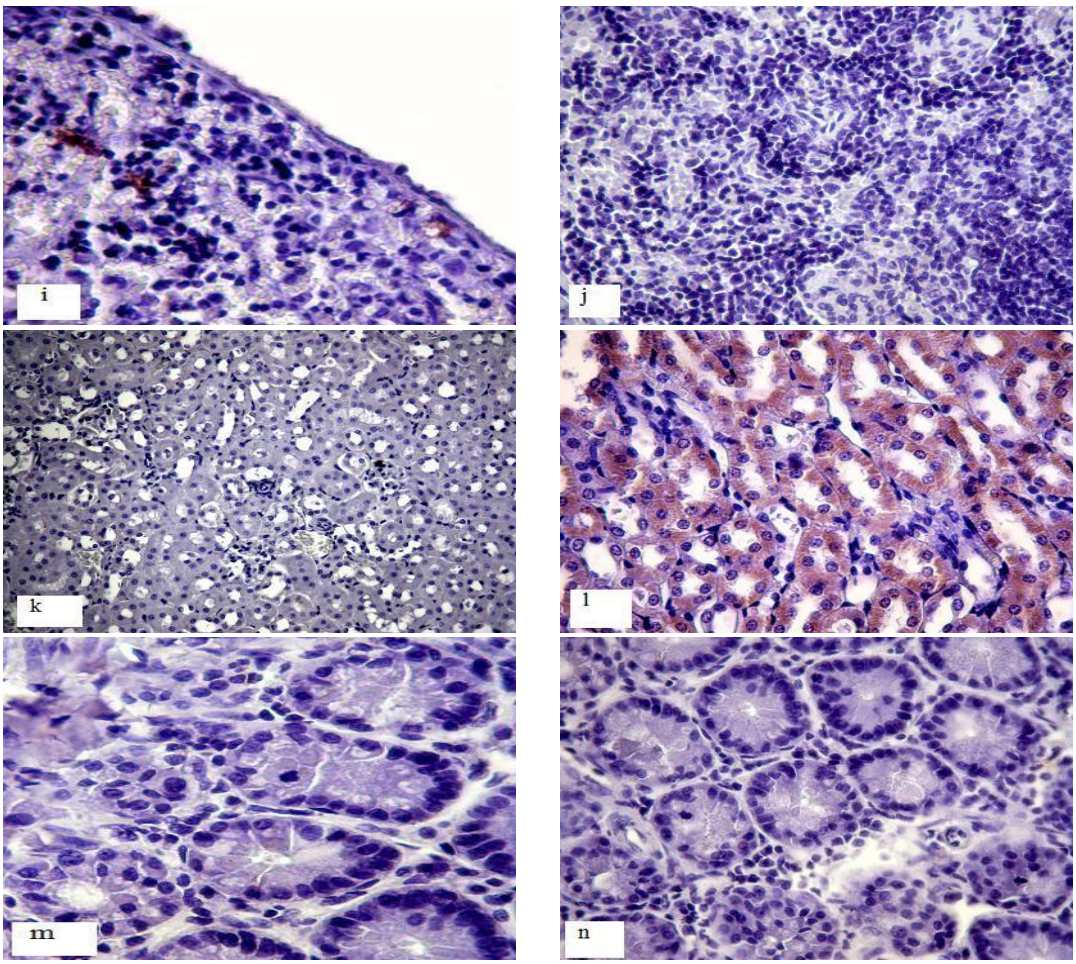

Figure 2. Continue.
Şekil 2. Devamı.

Table 1. The staining intensities of glutathione S-transferase alpha, pi in tissues of *Spermophilus xanthoprymnus and Meriones tristrami.*
Çizelge 1. Spermophilus xanthoprymnus ve Meriones tristrami dokularında glutatyon S-transferaz alfa, pi'nin boyama yoğunlukları.

Tissue types	Species	GST- alpha	GST- pi
Heart	S1	1	3
	S2	2	2
Kidney	S1	0	0
	S2	3	3
Liver	S1	1	2
	S2	2	2
Lung	S1	1	2
	S2	1	2
Small intestine	S1	0	0
	S2	0	0
Spleen	S1	2	1
	S2	2	0
Stomach	S1	1	2
	S2	0	0

A graphic showing the distribution of expressions of GST-Alpha and GST-Pi isozymes between *Spermophilus xanthoprymnus* (S1) and *Meriones tristrami* (S2) is given in Figure 3.

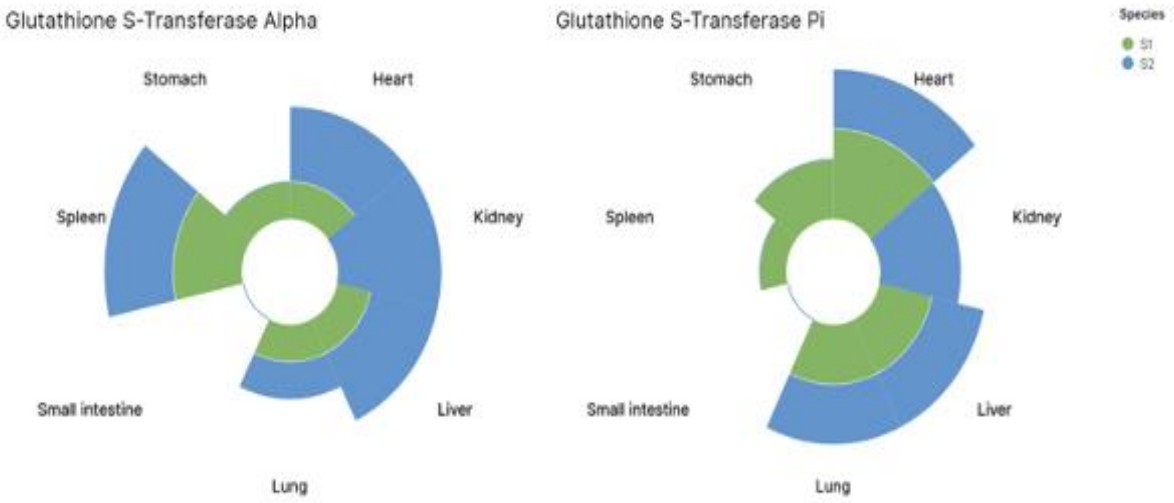

Figure 3. General distributions of expression rates of GST isozymes in *Spermophilus xanthoprymnus* and *Meriones tristrami* species after immunohistochemical analyzes.
Şekil 3. İmmunhistokimyasal analizler sonrasında Spermophilus xanthoprymnus ve Meriones tristrami türlerinde GST izozimlerinin ekspresyon oranlarının genel dağılımları.

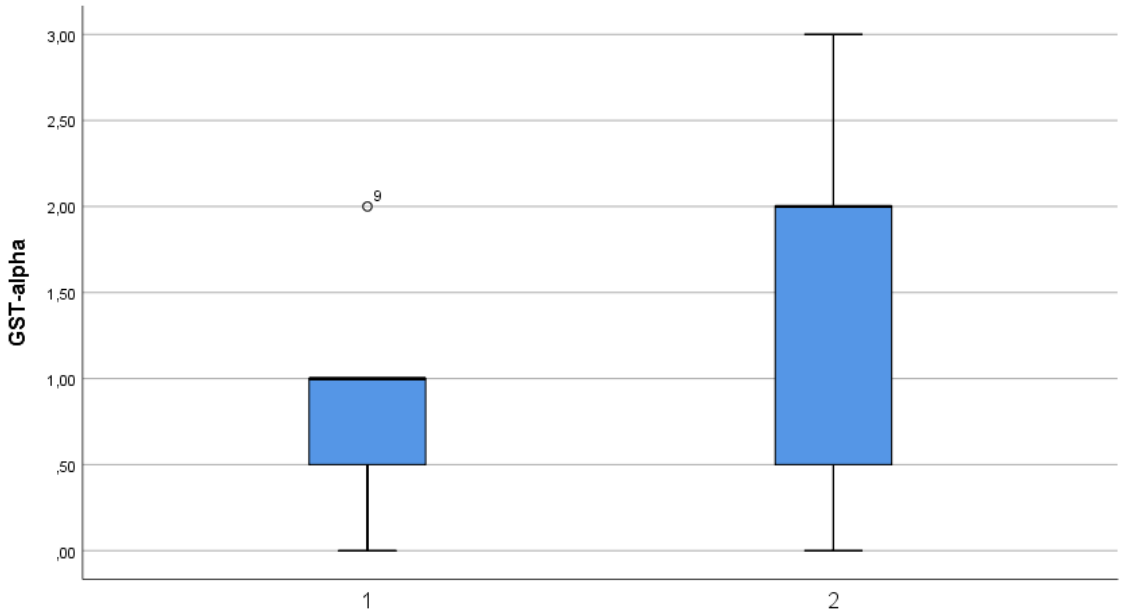

Figure 4. GST alpha expressions of species 1 and 2.
Şekil 4. Tür 1 ve 2'nin GST alfa ifadeleri.

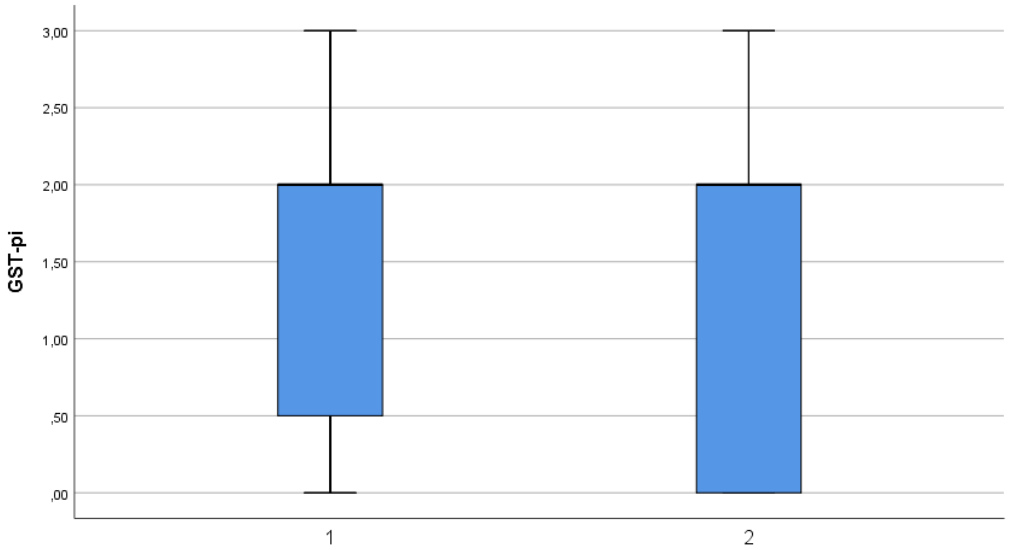

Figure 5. GST pi expressions of species 1 and 2.
Şekil 5. Tür 1 ve 2'nin GST pi ifadeleri.

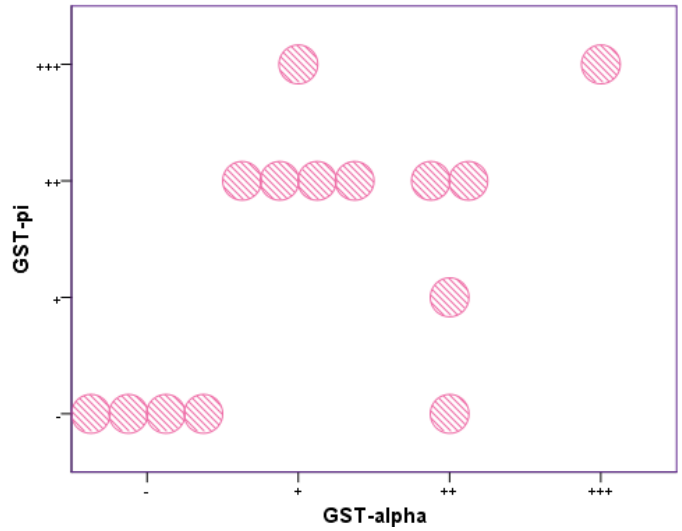

Figure 6. The relationship between GST alpha and GST pi expressions.
Şekil 6. GST alfa ve GST pi ifadeleri arasındaki ilişki.

DISCUSSION AND CONCLUSION

Many studies have demonstrated the effects of toxic substances various living beings are exposed to in their habitats, on their metabolism and the differences in the phase II phase of detoxification (Leblanc and Dauterman, 2001). They have revealed not only their sheltering environments, nutrition and relations with other living things, but also the effect of atmospheric toxicities and even the extent to which they are related to Phase II GSH expression levels in their habitats (Moran, 1995).

In some studies, GST-Pi isozymes were analyzed comparatively in order to compare the environmental exposure in rats, some fish species and human beings. In a study, using HPLC, western blot, immunohistochemistry and amino acid sequencing, when the amino acid sequencing results were analyzed in terms of GST-Pi enzymes, it was observed that there was 65% homology between rats and humans (Avcı et al., 2014).

While GSTP1 can be isolated from some human tissues, it is abundant especially in mammalian erythrocytes. Since the isozyme that is active in Phase II enzyme activation in mammals is generally focused on GSTP1, it is thought to be more related to this isozyme. Various methods can be used to determine its

identification and amount and carry out its isolation and analysis. Studies assessing the results of meta-analysis studies have reported that the GSH ratios and expressions of mammals living in similar or different habitats can be expressed in relation to detoxification and toxic composites in their habitats (Casalino et al., 2004).

GST isozymes are thought to be involved in cell signaling and fertilization in male mammals. Especially for isozymes such as GSTM3, GSTO2, GSTM1 and GSTT1, if their metabolic expression exhibits similar effects across mammals, then that will adversely affect the fertilization of mammalian species. And as a result, it can be thought that it will contribute to a decrease in their numbers or even extinction in their natural environment (Rowsey et al., 2001).

GSH enzymes prevent cell damage by inactivating and eliminating electrophilic mutagenic and carcinogenic compounds. In a study conducted on dogs (*Canis lupus*), it was reported that GST-Pi in humans was heterologous with CluGST-Pi in dogs and had a cross reactivity. There was an important mechanism against similar anti-carcinogenic toxic elements in the mechanism of tumor formation. When many toxic materials in the substrate were examined with GST-Pi, it was reported that especially benzyl isothiocyanate was the most active substrate (Park et al., 2005).

In a study conducted in Central Anatolia in 2014 to reveal how hibernation affects the *Spermophilus xanthoprymnus* species and determine the oxidation and anti-oxidant states in metabolism of this animal, nitrogen oxide species (NOx) and malondialdehyde (MDA) levels of 9 female ground squirrels were determined by spectrophotometric analysis method, and GSH levels were found to be lower in the hibernation group than in the awakened group (Inoue et al., 1999).

When the studies on *Meriones tristrami*, the other species studied in the present study, were examined, it was found that in these studies, it was applied as a food at different concentrations to the feeds used in these animals by examining the increase in the expression of detoxification enzymes. When these structures were eliminated, GSH enzyme levels were observed to decrease rapidly (Egaas et al., 1993).

In the literature, the metabolism of these two species, which are the subject of our study, depending on the GSH and detoxification mechanism and the situation of these species, which have similar living conditions, have rarely be studied. In order to reveal the toxic exposures of these species in their natural environments, GST-Pi expression levels are determined by tissue follow-up and immunohistochemical method on samples taken from various organs. Furthermore, GST-Pi is the focal point in terms of isozymes in the detoxification mechanism in mammals and other living species. We also evaluated the GST-alpha levels by comparing two isozymes. Quite different results were found between these species in organs that differed in intensity of enzyme expression. In terms of kidney tissues, GST alpha expression was not found in *Spermophilus xanthoprymnus*, but was strongly expressed in *Meriones tristrami*. Moderate expression of GST alpha was observed in stomach tissue of *Spermophilus xanthoprymnus*, whereas GST alpha expression was not observed in stomach tissue of *Meriones tristrami*. When kidney tissues were examined, the expression rate of GST pi was quite strong for *Spermophilus xanthoprymnus*. On the other hand, no expression was observed in kidney tissue of *Meriones tristrami*. GST Pi expression was not observed in stomach and spleen tissues in *Meriones tristrami*, whereas moderate GST Pi expression was observed in *Spermophilus xanthoprymnus*.

The results of the study showed that the enzymes involved in the detoxification mechanism were observed at different rates in various organs of *Spermophilus xanthoprymnus* and *Meriones tristrami* living in the similar habitat under similar natural conditions, along with the expected results of exposure to toxic substances. In addition to being dependent on the toxicity in the habitat, this situation may cause these results to occur due to the rapidly increasing use of pesticides in agriculture and the nutritional habits of these creatures. It is very important in terms of the mutagenic effect and the continuation of the generations whose fertility may occur with this toxic exposure, together with the increased GSH status, by using more advanced techniques in the future studies. It is recommended to investigate whether exposure to toxicity has an effect

on the reduction of their prevalence and numbers in nature in both types, and to take measures to preserve these species for this purpose.

As a result, glutathione s-transferase-alpha and glutathione s-transferase-pi expression levels were found to differ in *Spermophilus xanthoprymnus* and *Meriones tristrami* samples obtained from different localities of Kırıkkale province.

ACKNOWLEDGEMENT

We are thankful for Aykut Arif Topçu and İrfan Albayrak for their contribution to this study.

AUTHOR CONTRIBUTION

The authors declare that they have contributed equally to the article.

FINANCIAL SUPPORT

This research received no grant from any funding agency/sector.

ETHICAL STATEMENT

Ethics committee approval of Kırıkkale University Animal Experiments Local Committee was received for this research study (Approval No: 2019-6-35). Legal permissions for this study were provided by The Turkish Ministry of Agriculture and Forestry, General Directorate of Nature Conservation and National Parks (Türkiye) with the document dated 16.08.2019 and numbered 21264211-288.04- E.2467643.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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