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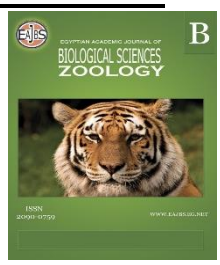

Melanomacrophage Centers: Comparative Histological Insights among Three Reptilian Species

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ABSTRACT

Background: Reptiles have been suggested to be a suitable model for the histological and histochemical aspects even better than other used experimental mammals**.** Melanomacrophage centers (MMCs) are aggregations of macrophage-like cells and pigments including melanin, hemosiderin, and lipofuscin. MMCs found mainly in different tissues of non-mammalian vertebrates. MMCs are helpful biomarkers for a variety of stresses as environmental pollutants. **Aim:** This study was designed to elucidate the histological, morphometric, histochemical and immunohistochemical characterization of hepatic MMCs in three reptilian species; *Uromastyx ornata* (Ornate Dabb lizard), *Uromastyx aegyptiaca* (Egyptian Dabb lizard), and *Varanus griseus* (Desert Monitor lizard). **Methods:** Fifteen adult male reptilian animals were divided into; i. Five adult males Ornate Dabb lizard, ii. Five adult males Egyptian Dabb lizard, iii. Five adult males Desert Monitor lizard. Liver paraffin sections of all animals were processed and stained with Hematoxylin and Eosin stain, Masson Trichrome stain for collagen fibres and Perl's Prussian Blue histochemical stain for hemosiderin pigments. Immunohistochemical demonstration of melanin pigments via HMB-45 and macrophage lineage via CD68 immunostains were applied. Histomorphometric assessments of MMCs were also performed. **Results:** Histomorphometric examinations revealed significant differences between the histomorphometric structures of MMCs among the experimented species. MMCs in Desert Monitor lizard were fewer in number and smaller in size; with decreased melanin pigment contents and macrophage lineages, and increased hemosiderin contents. **Conclusion:** The significant differences in MMCs among the three reptiles may indicate considerable differences between the immune system of the Desert Monitor lizard and each of the Ornate and Egyptian Dabb Lizards.

INTRODUCTION

Reptiles are the most prevalent vertebrates in deserts, where they occupy almost every imaginable environment. Because of their bodies' overlapping keratin scales, lizards can survive in the driest deserts on the earth (Akiyoshi and Inoue, 2012, Rutland *et al.*, 2019). Though numerous reptilian species are found in or near water, they do not lay eggs underwater (Akiyoshi and Inoue, 2012; Allam *et al.*, 2016). Reptiles are classified as amniotes, along with birds and mammals (Akiyoshi and Inoue, 2012), and they are classified into four orders: Crocodilia (alligators and crocodiles), Chelonia (tortoises and

turtles), Rhynchocephalia (tuatara) and Squamata (lizards, snakes and worm-lizards). Squamates are the largest reptile order and are subdivided into Lacertilia (lizards and amphisbaenians) and Ophidia (snakes) (Chang *et al.*, 2009; and Rutland *et al.*, 2019). With the exception of some snake species, which are legless, lizards are quadrupedal squamates. Lizards are frequently territorial and have a variety of predator defense mechanisms, including camouflage, venom, and reflex bleeding. Though some reptiles such as land tortoises, some turtles, and some lizards are herbivorous, the majority of reptiles are primarily carnivorous or insectivorous. However, compared to what is required for birds and mammals, the overall amount of food consumed by reptiles is modest (Zaher *et al.*, 2012).

Uromastyx aegyptiaca, also known as the Egyptian Dabb lizard or spiny-tailed lizard, is a species of lizard that belongs to the genus *Uromastyx*, family Agamidae, suborder Iguania, order Squamata, and class Reptilia. It is present across North Africa, particularly in Egypt. Being a purely herbivorous species, *Uromastyx aegyptiaca* is one of the very few lizard species to have this feeding habit (Zaher *et al.*, 2012; Kandyel *et al.*, 2021). *Uromastyx ornate*, or the ornate Dabb lizard, is an herbivorous lizard species that also belongs to the genus *Uromastyx*, is a species of herbivorous lizard that is native to the Middle East and lives in rocky terrain in Yemen, Saudi Arabia, and Egypt. Given that various sexes have varied body colors, they are sexually dichromatic. While females of the *Uromastyx ornate* are paler, with a light brown back and light-yellow transverse lines, males of the species have a back that is primarily green, blue, or red with dark brown bands that may be filled with yellow spherical dots (Baha el Din, 2006; Wilms and Böhme 2007). *Varanus griseus*, often known as the Desert Monitor lizard, is a species of lizards that belongs to *Varanus* griseus, family Varanidae, order Squamata, and class Reptilia. It is endemic to Southwest Asia and North Africa. It inhabits sandy areas; like vast wadis and desert plains that have some vegetation. The Desert Monitor lizard is a sizable, diurnal carnivore that consumes various invertebrates, rodents, snakes, and smaller lizards. It can also consume an Egyptian Dabb lizard with comparable body size. During its foraging activity, it traverses quite a huge area (Ibrahim, 2010; Soliman and Mohallal, 2016).

Modern reptiles have a digestive system that is structurally comparable to that of all higher vertebrates, as it consists of the buccal cavity with its associated structures, the esophagus, the stomach, the small and large intestine, as well as the accessory digestive glands as the liver and the pancreas (Kardong, 2012; Zaher *et al.*, 2012; Srichairat *et al.*, 2022). Since reptiles respond to food even better than other regularly used experimental mammals like mice, rats, rabbits, and pigs, they have been suggested to be a suitable model for investigating the physiological regulation of the digestive process in the future (Mohammed *et al.*, 2021).

Compared with mammals, reptiles possess a variety of peculiarities that could be of specific advantages for some histological and histochemical aspects (Zaher *et al.*, 2012). In reptiles, the liver is the largest extrinsic digestive gland where different substances are taken in by intestinal capillaries and carried into the hepatic portal vein by tributaries, where the first processing. Depending on the visceral cavity, the reptilian liver can adopt a range of morphologies. While it is thicker and more compact in numerous reptile species, it is lengthy and thin in several lizard and snake species (Nafady and Awadalla, 2019).

Melanomacrophage centers (MMCs) are unique aggregations of macrophage-like cells, which are fragments derived from phagocytosed cells that were mostly composed of erythrocytes and pigments including melanin, hemosiderin, and lipofuscin (Dang *et al.*, 2021; Viana *et al.*, 2021). MMCs are found mainly in the reticuloendothelial tissue of haemolymphopoietic organs including the liver, kidney, spleen and pancreas of different non-mammalian vertebrates like reptiles (Domiciano *et al.*, 2017), amphibians (Wu *et al.*,

2017) and fishes (Sales *et al.*, 2017). In fishes, MMCs are occasionally located in the thymus, intestinal submucosa, gills, brain, and gonads (Basilone *et al.*, 2018; Stosik *et al.*, 2019; Dang *et al.*, 2021, Viana *et al.*, 2021). MMCs are involved in the detoxification, recycling, and elimination of both endogenous and external elements, including cell debris and dead cells. MMCs are helpful response biomarkers for a variety of stresses, such as environmental pollutants. Additionally, MMCs have been applied as biomarkers to evaluate the health of fish and the contamination of the aquatic environment. MMCs have been considered metabolic dumps that contain both endogenous and exogenous materials including antigens, and biomarkers for exposure to various anthropogenic stressors (Qualhato *et al.*, 2018; Dang *et al.*, 2021; Viana *et al.*, 2021).

In an attempt to correlate histology with ecology and environmental sciences, as well as maximize the role of histology and histochemistry which may contribute to climate change studies in the future, this study was designed to elucidate the histological, morphometric, histochemical and immunohistochemical characterization of hepatic MMCs in three reptilian species; *Uromastyx ornate, Uromastyx aegyptiaca* (Herbivorous reptiles), and *Varanus griseus* (Carnivorous reptile) in a comparative manner.

MATERIALS AND METHODS

Animals and Samples Collection:

A total of 15 males' adult reptilian animals were captured in the summer season - July 2021, and were divided into three groups as follows:

1- Five adult *Uromastyx ornate* males (Ornate Dabb lizard) weighting 150-220g and 35-40 cm in length, captured from Sharm El Sheikh, South Sinai, Egypt, were used as a model of herbivorous reptiles.

2- Five adult *Uromastyx aegyptia aegyptia* males (Egyptian Dabb lizard) weighting 345-365g and 40:50 cm in length, captured from Gebel El-Maghara Area, North Sinai, Egypt, were used as a second model of herbivorous reptiles.

3- Five adult *Varanus griseus* males (Desert Monitor lizard); weighting 750-775 g and 85:95 cm in length, captured from El Dabaa, Marsa Matrouh, Egypt, were used as a model of carnivorous reptiles.

All of the collected reptile species were sacrificed, and dissected according to Suez university animal care and use committee guidelines. A longitudinal incision was made at the midventral surface (**Fig. 1**), and small pieces of the hepatic tissues were removed and prepared for further examination.

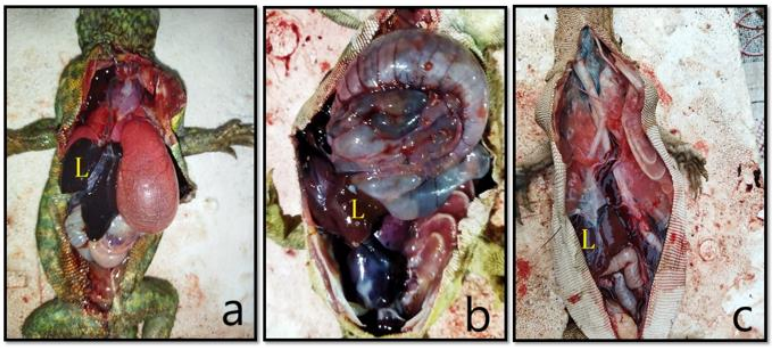

Fig.1: Photo of the anatomy of the three reptilian species; a: *Uromastyx ornate* (Ornate Dabb lizard; b: *Uromastyx aegyptia* aegyptia (Egyptian Dabb lizard); c: *Varanus griseus* (Desert Monitor lizard); L: liver.

1- Histological Studies:

Small specimens of the livers of all experimental reptiles were fixed in a

neutral 10% formalin solution, then washed, dehydrated, cleared in xylene, and impregnated in paraffin wax for blocking. Five µm thick sections were prepared using a rotary microtome, and then sections were stained by:

1. Hematoxylin and Eosin (H&E) stain for microscopic examination of hepatic tissues (Bancroft and Gamble, 2008).
2. Masson trichrome stain to evaluate collagen fibers accumulation (Bancroft and Gamble, 2008).

2- Histochemical Demonstration of Hemosiderin:

Five µm thick paraffin sections were stained with Perl's Prussian Blue (PPB) stain for hemosiderin pigments (Ellis, 2007).

3- Immunohistochemical Studies:

Avidin-biotin peroxidase complex method was used in immunohistochemical examinations by following the manufacturer's instructions. Briefly, paraffin sections of 4-µm thickness were deparaffinized on charged slides, cleared with xylene, rehydrated in a gradient ethanol series, and washed with distilled water. Then, sections were heated in a 10% citrate buffer solution and allowed to cool at room temperature for 20 mins. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and proteins Block Serum-Free (Dako, Japan) was applied to prevent nonspecific protein binding. Microwave-assisted antigen retrieval was performed for 20 min. Sections were incubated overnight at 4ºC with the primary antibody of:

i. Anti-HMB45: For identification of melanin pigments, four µm thick paraffin sections were stained with Monoclonal Mouse Anti-Human Melanosome antibodies, (Code M0634, Dako, Copenhagen, Denmark).

ii. Cluster of Differentiation-68 (CD68): To identify cells of macrophage lineage, four µm thick paraffin sections were stained with macrophage markers CD68 Ab-4 (mouse monoclonal antibody, Thermo Scientific, code MS-1808-S0).

Then, sections were washed in PBS for five min, incubated with ready-to-use Biotinylated Goat-anti-rabbit immunoglobulin secondary antibody (#BP-9100; undiluted; Vector Laboratories, Burlingame, CA, USA) for 20 min at 30˚C, and finally washed in PBS for 5 min and incubated with the peroxidase detection system Ready to Use conjugated antibody (#RE7110-K; Novocastra; Leica Microsystems, Inc.) for 20 min. Sections then were rinsed with PBS for 5 min, incubated with chromogenic 3,3'-diaminobenzidine (DAB) (Leica Microsystems, Inc.) for 5 min, washed with tap water and counterstained with hematoxylin; all steps were completed in room temperature (Ramos-Vara et al., 2008).

4- Quantitative and Histomorphometric Evolutions and Images Analysis of MMCs:

Histomorphometric analyses of MMCs were performed in images obtained from five non-overlapping (10×) randomly selected fields from each hematoxylin and eosin (H&E) stained slide, using image analyzer software (ImageJ program; version 6.0; Media Cybernetics Inc., Bethesda, Maryland, USA), then the average was recorded. For this purpose, several histomorphometric parameters were estimated: (i) size (perimeter) of MMC (µm2); (ii) number of MMCs per total field area; (iii) individual MMC perimeter; (iv) Total MMC covered area (%). Furthermore, Percent (%) of each of Pearl's positive stained areas, HMB-45 positive reaction-stained area and CD68 positive reaction-stained area were assessed on images obtained from five non-overlapping (40×) randomly selected fields.

5- Statistical Analysis:

Statistics were calculated using the student's T-test and SPSS for windows v.16. Results were presented as mean ± standard division (SD), and all statistical comparisons were analyzed by means of a one-way ANOVA test followed by Post hoc analysis. A (P

< 0.01) value was considered as significant difference**.**

RESULTS AND DISCUSSION

1- Histological Observations:

Histological inspections of the liver section of both Ornate and Egyptian Dabb lizards revealed nearly the same histological structure (Figs 2&3). The liver is surrounded by a thin hepatic capsule (Glisson's capsule) which is prominent in all higher vertebrates and contributes to hepatic parenchyma division into structural hepatic lobules but compared with mammals, where the liver is not as organized into lobules distinctly. The hepatocytes are polygonal in shape and arranged in cords separated by a thin connective tissue and exhibit a net-like structure arising from a centrally located central vein to the lobular periphery. Hepatic cords' radial arrangement in relation to the central vein is typically obscure. Plates of hepatic cells are separated by a network of short and narrow blood sinusoids which are lined with endothelial cells and contain Kupffer cells. The hepatocytes contained a homogenously fine granulated cytoplasm, embodying eccentric located rounded nuclei. The lumen of the portal vein appears to enclose a few blood cells. The bile ducts are surrounded by a layer of cuboidal epithelium. Elliptical-shaped red blood cells (RBCs) with oval nucleus were observed. Impregnation with Masson trichrome stain revealed that the hepatic tissue is supported with delicate collagen fibers that surround the hepatic cells and veins, while sinusoids represented a mild amount of collagen fibers stroma. Moreover, each portal area was surrounded by a collagenous network which was continuous with a moderate amount of scanty interlobular collagenous fibers.

Similar histological structures of different lizards were reported such as *Acanthodactylus boskianus* lizard (Nafady and Awadalla, 2019), *Tropidurus torquatus* lizard (Firmiano *et al.,* 2011), *Tritures karelinií* and *Tritures vulgaris* (Koca *et al.,* 2004), Qinghai Lizard (*Phrynocephalus vlangalii*) (An and Zhang, 2019), and freshwater turtle *Phrynops geoffroanus* (Moura *et al.,* 2009).

Histological examination of the hepatic tissue of *Varanus griseus* (Desert Monitor lizard) (Fig. 4) revealed a thicker connective tissue capsule surrounding the liver parenchyma when compared with that of ornate and Egyptian Dabb lizard. Through the hepatic tissue, the central veins, sinusoids, and portal veins were randomly distributed. Simple cuboidal epithelium lined the bile ducts that were situated close to portal veins. The hepatocytes were polyhedral in form and had heavily vacuolated cytoplasm, as many cells displayed fat drops in their cytoplasm. Each hepatocyte has a single eccentric, vesicular nucleus that is spherical including the prominently dark nucleolus. The hepatocytes were arranged as glandular-like or alveolar-like groups, and each was encircled by a network of sinusoidal capillaries of various sizes. In the sinusoidal lumen or connected to their surface, phagocytic Kupffer cells could be detected. The sinusoidal spaces were filled with a large number of elliptical erythrocytes with oval nuclei. Masson trichrome stained sections revealed a densely stained collagenous network that forms a thick hepatic capsule, supports hepatic parenchyma, and surrounds hepatic central and portal veins, and blood sinusoids. There was a marked increase of collagen-stained area (%) in the hepatic tissues of the Desert Monitor lizard when compared with both Ornate and Egyptian Dabb lizards. Similar histological features in Nile monitor (*Varanus niloticus*) were reported (Ahmed et al., 2018). The hepatic parenchyma of the Nile monitor was not arranged in the well-known classical manner of the central vein, hepatic plates, sinusoids and portal area, as the central vein, sinusoids and portal area were haphazardly organized. Additionally, the hepatocytes appeared vacuolated and alveolar groups were

surrounded by twisted sinusoidal networks. This hepatic histoarchitecture is not unique to the Desert and Nile monitor but was of a similar histological structure to the liver of turtles (Mezyad, 2015; Moura *et al.* 2009). In accordance with our results, the hepatocytes of the Nile monitor revealed a high number of lipid droplets (Ahmed *et al.*, 2018).

Unlike mammalian liver, such species' substantial lipid storage shouldn't be considered a pathogenic state. The type of food this species consumes, which includes invertebrates and small vertebrates, is probably responsible for the liver's high lipid concentration (King and Green 1999). In our study, the specimens were collected throughout the summer, a non-hibernating season in Egypt; this may explain this feature as it is widely known that hibernating animals have higher levels of lipogenesis enzymes in the summer than in the winter. (Anderson *et al.*, 1989; Wang *et al.*, 1997). The Nile monitor lizards' hepatocytes store lipids in the summer for hydrolysis as an energy source when they hibernate in the winter. (Ahmed *et al.*, 2018). Characteristic nucleated oval RBCs were also observed filling the sinusoids in the hepatic tissue of the Nile monitor (Ahmed *et al.*, 2018). RBCs are recognized for carrying oxygen, but they are also now thought of as one of the non-immune cells that support the immune response against foreign hazardous chemicals. (Passantino *et al.*, 2007).

Melanomacrophage centers (MMCs) were recorded in the hepatic parenchyma in all three reptiles, Ornate Dabb lizard, Egyptian Dabb lizard and Desert Monitor lizard, with a variance of shape, localization, distribution and density, as described in the following:

2- Comparative Histological and Quantitative Analyses of MMCs:

i. Distribution:

In hepatic tissues of both Ornate Dabb lizards and Egyptian Dabb lizards, MMCs appeared as clumps of cells with dark brown color, as they were densely located around blood vessels, especially the portal veins, then they were found less densely around the central vein and slightly in the peripheral regions. In the Desert Monitor lizard, MMCs existed as a few pigmented cells forming a part of the lining of the sinusoids, and protruded into the sinusoidal lumen; they were also occasionally seen in the hepatic parenchyma (Figs. 2-4).

ii. Morphological Features:

Shape: Hepatic MMCs in Ornate Dabb lizards and those in Egyptian Dabb lizards were round, oval, or polymorphic in shape, while MMCs in hepatic tissues of Desert Monitor lizard were all polymorphic in shape (Figs. 2-4; Table **1**).

Structure: In hepatic tissues of Ornate Dabb lizards, well-structured with high-intensity MMCs were often recorded more than those of Egyptian Dabb lizards which were partially structured centers. However, MMCs in both Ornate Dabb lizards and those in Egyptian Dabb lizards revealed a well-defined membrane. MMCs of Desert Monitor lizard appeared as irregular unstructured bodies without any surrounding membranes and with low intensity (Figs. 2-4).

Size: On average, hepatic MMCs in Ornate Dabb lizards were significantly larger ($P < 0.01$) than those in Egyptian Dabb lizards and Desert Monitor lizards which were both smaller in size, however, MMCs in Desert monitor lizards were significantly smaller ($P < 0.01$) than those of Egyptian Dabb lizards (Figs. 2-4; Table 1).

Individual MMC covered area (%): In relation to MMCs size, the area covered with individual MMC was significantly ($P < 0.01$) increased in Ornate Dabb lizards when compared with those in Egyptian Dabb lizards and in Desert monitor, which in turn occupied the smallest area.

Color: In H&E stained sections, hepatic MMCs in Ornate Dabb lizards and those in Egyptian Dabb lizards appeared as dark masses condensed with deep brown color, while

MMCs in Desert monitor hepatic tissues were in yellow color and sometimes difficult to recognize (Figs. 2-4; Table 1).

iii. Variation in Abundance:

Prevalence (% of animal population): MMCs were observed in all examined hepatic tissues (100%) in Ornate Dabb lizards, Egyptian Dabb lizards and in Desert monitor at a decreasing order of prevalence (Figs. 2-4; Table 1).

Numbers*:*MMCs were common in the liver of both Ornate Dabb lizards and in Egyptian Dabb lizards with an insignificant difference in number, while MMCs were rare in Desert monitor liver with a significant decrease ($P < 0.01$) as compared with both Ornate Dabb lizards and in Egyptian Dabb lizards ((Figs. 2-4; Table 1).

Total MMCs Covered area (%): In relation to size and individual MMC occupied area, hepatic MMCs in Ornate dabb lizards recorded the highest total MMCs covered area (%) ($P < 0.01$) when compared with the other two animals. Then, at a decreasing order of prevalence, Egyptian Dabb lizards and Desert monitor (Figs. 2-4; Table 1). In the hepatic tissue of the Nile monitor (*Varanus niloticus*), MMCs were occasionally seen in sinusoidal spaces within small size (Ahmed *et al.*, 2018), which may be in accordance with our findings related to Desert Monitor Lizard. Melanomacrophage centers were observed in different reptilian species, especially lizards, by several authors. In hepatic tissues of *Tropidurus torquatus* lizard, a large quantity of well-structured MMCs was indicated as distinctive groupings of pigment-containing cells, which increased in the portal area (Firmiano *et al.*, 2011). An and Zhang, (2019) distinguished un-structured MMCs in the hepatic parenchyma of Qinghai Lizard (*Phrynocephalus vlangalii*) which were rich in melanin pigments. MMCs aggregates contained approximately equal proportions of melanin and hemosiderin granules in the center of the portal tract in the hepatic parenchyma of freshwater turtles (*Trachemys scripta*) (Divers *et al.,* 2010). Red-eyed crocodile skinks (*Tribolonotus gracilis*) appeared in two forms, large polymorphic MMCs aggregates and small diffused macrophages scattered all over the hepatic tissue (Kwon *et al.*, 2019). Akiyoshi *et al.* (2015) designed a study on the livers of 23 reptilian species and they revealed the distribution of MMCs in different zones in hepatic parenchyma. MMCs, appeared in yellow to brown compounds, lining the sinusoids and protruding into the sinusoidal lumen. In the order Testudines, the distribution of MMCs were observed in sinusoidal capillaries, and various sizes existed. In the order Squamata, MMCs were observed in the suborder Lacertilia, but not in the suborder Serpentes, except for the genus Hydrophis, the Black-headed Sea snake, where the MMCs were fewer in number and smaller in size in the sinusoids. In the suborder Lacertilia, the MMCs were distributed in sinusoidal capillaries and in portal tracts, and the size of MMCs was small. In the genera, *Hemidactylus* and *Plestiodon*, the distribution of MMCs were in all hepatic regions, but MMCs were larger and widely observed in the sinusoidal capillaries. In the order Crocodilia, MMCs were of moderate size in sinusoidal capillaries (Akiyoshi *et al.*, 2015). The phylogenetic order of the MMCs was however influenced by the number, sizeable, and locational heterogeneities, according to Akiyoshi *et al.* (2015). Variations in the number of MMCs, their size and distribution vary according to species, organ, age, nutritional status and environmental and stress conditions (Ribeiro *et al*., 2011; Pronina *et al*., 2014). Based on the presence or absence of a membrane, which consists of a thin single layer of flattened cells, MMCs were classified basically into well-structured or unstructured (Qualhato *et al.*, 2018).

Table 1: Histomorphometric and Quantitative Analyses of Hepatic melanomacrophage centers (MMCs) among Ornate Dabb lizard, Egyptian Dabb lizard and Desert Monitor lizard *(n=5 ; P <0.01).*

Parameter \ animal		Ornate Dabb Lizards	Egyptian Dabb Lizards	Desert monitor
Morphology	Shape	Round, oval or polymorphic	Round, oval or polymorphic	Polymorphic
	Size (perimeter)	2.22±0.37	1.32±0.22[a]	0.48±0.07[a, b]
	Individual MMC Area (%)	0.36±0.087	0.15±0.026[a]	0.014±0.002[a, b]
	Color (HE)	Brown	Brown	Yellowish brown
	Structure	Well structured	Partially structured	Un-structured
	Membrane	Yes	Yes	No
Distribution		Portal area	Portal area	Embedded within sinusoids
Abundance	Prevalence of animal population (%)	100%	100%	100%
	Number / field	28.60±1.67	26.20±1.92	7.60±0.54[a,b]
	Total MMC covered area (%)	24.08±3.02	8.26±1.55[a]	2.87±0.70[a,b]
Prevalence of MMCs with pigments (%)	Hemosiderin (PP(	0.66±0.20	1.14±0.064	7.11±0.70[c,d]
	Melanin (HMB-45) (%)	20.21±1.60	4.33±0.70[a]	1.15±0.10[a,b]
Lineage with macrophages	(CD68%)	12.37±1.50	3.70±0.25[a]	1.63±0.19[a, b]

(a): Significant decrease when compared with Ornate Dabb lizard; (b): Significant decrease when compared with Egyptian Dabb lizard; (c): Significant increase when compared with Ornate Dabb lizard; (d): Significant increase when compared with Egyptian Dabb lizard.

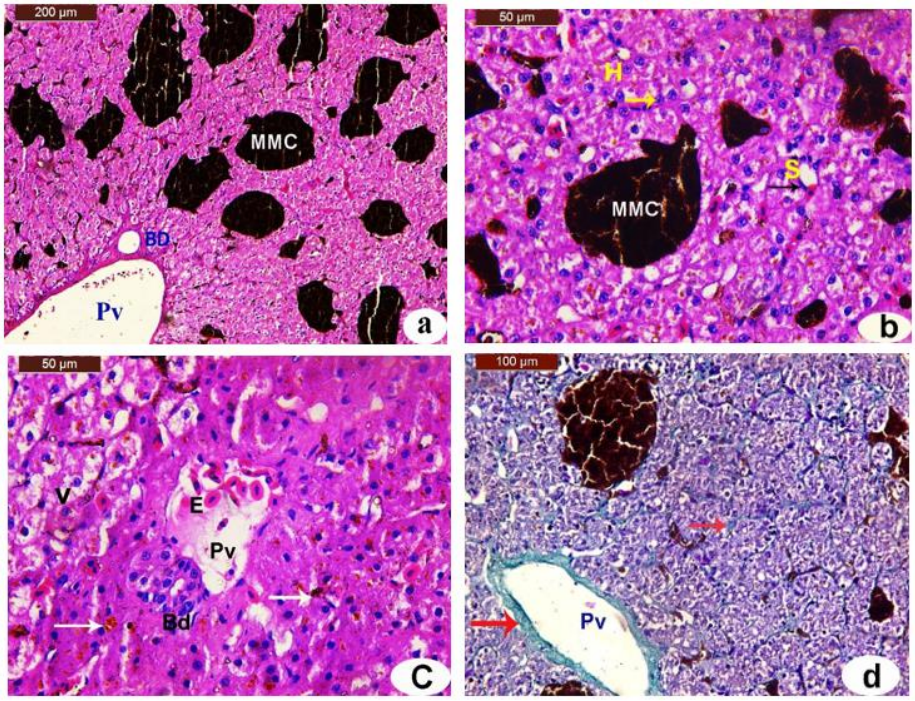

Fig. 2: Histoarchitecture of hepatic tissue of Ornate Dabb Lizard. **(a)** Liver section showing portal vein (Pv), bile duct (Bd), and numerous large dark melanomacrophage centers (MMC) (H&E stain; scale bar 200µm). **(b)** Liver section showing polygonal hepatocytes (H), with eccentric rounded nucleus (yellow arrow), sinusoids (S) with Kupffer cells (black arrow) (H&E stain; scale bar 50µm). **(c)** Liver section showing hepatocytes with vacuolated cytoplasm (v), Elliptical-shaped erythrocytes with oval nucleus (E), bile duct lined with cuboidal epithelium (Bd) and few scattered MMCs (white arrow) (H&E stain; scale bar 50µm). **(d)** Liver section showing greenish blue collagen fibers surrounding portal vein (Pv), and separating hepatocytes plates (red arrows); (Masson trichrome stain; scale bar 100µm).

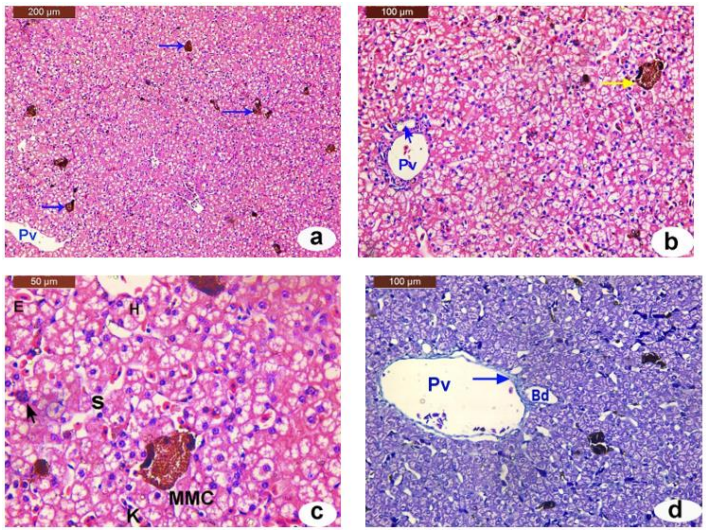

Fig. 3: Histoarchitecture of hepatic tissue of Egyptian Dabb Lizard. **(a)** Liver section showing portal vein (Pv), and few and small dark melanomacrophage centers (arrows) (H&E stain; scale bar 200µm). **(b)** Liver section showing granular MMC (arrow), portal vein (Pv), bile duct with cuboidal lining epithelium (arrowhead) (H&E stain; scale bar 100µm). **(c)** Liver section showing hepatocytes with vacuolated cytoplasm (H), epileptic nucleated oval erythrocytes (E), sinusoids (S) with Kupffer cells (k) and Heterophil cell (arrow) (H&E stain; scale bar 50µm). **(d)** Liver section showing greenish blue collagen fibers surrounding portal vein (Pv), (Masson trichrome stain; scale bar 100µm).

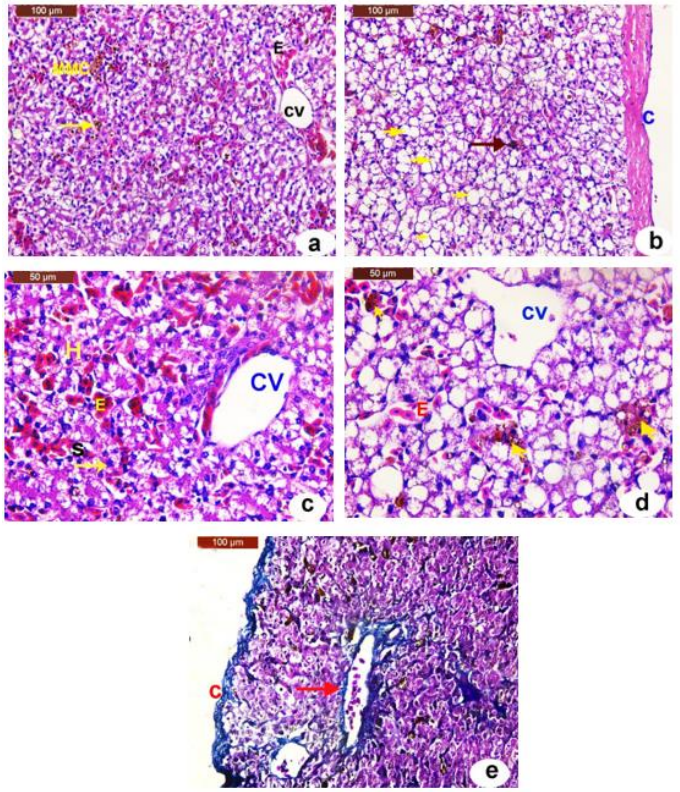

Fig4: Histoarchitecture of hepatic tissue of Desert Monitor Lizard. **(a)** Liver section showing central vein (cv), Elliptical-shaped erythrocytes with oval nucleus (E), and few and small pale melanomacrophage centers (MMC, arrows) (H&E stain; scale bar 100µm). **(b)** Liver section showing thick fibrous capsule (c), small MMC (brown arrow), fat droplets in hepatocytes cytoplasm (yellow arrows) (H&E stain; scale bar 100µm). **(c)** Liver section showing central vein (cv), hepatocytes (H), Elliptical-shaped with oval nucleated oval erythrocytes (E), and sinusoids (S) with Kupffer cells (arrow) (H&E stain; scale bar 50µm). (**d)** Higher magnification power of liver section showing small pale melanomacrophage centers (arrows), fat droplets in hepatocytes cytoplasm (H&E stain; scale bar 50µm). **(e)** Liver section showing dense greenish blue collagen fibers surrounding hepatic parenchyma forming liver capsule (c) and blood vessels (arrow), (Masson trichrome stain; scale bar 100µm).

4. MMCs Pigmentation:

Overall, the three reptilian animals, Ornate Dabb lizard, Egyptian Dabb lizard and Desert Monitor lizard exhibited positive histochemical reactivity with hemosiderin, and positive HMB-45 immunoreactivity for melanin, with considerable variations as follows:

i. Hemosiderin:

Perls' staining demonstrated that the MMCs in all the groups contained Hemosiderin. MMCs in Desert monitor lizards revealed the highest content of Hemosiderin when compared with the other two animals ($P < 0.01$), as each MMC appeared as an aggregation of hemosiderin particles. Each of the MMCs in livers of Ornate Dabb lizards and Egyptian Dabb lizards revealed traces of hemosiderin pigments as scant pale cytoplasm contained numerous Prussian blue positive hemosiderin granules, which were sometimes observed only in some single hepatocytes without any significant differences in hemosiderin contents between Ornate Dabb lizards and Egyptian Dabb lizard ($P < 0.01$) (Fig 5, Table 1).

In ectothermic vertebrates (amphibians, reptiles and fish), MMCs were reported to contain pigments such as melanin, hemosiderin and lipofuscins (Sales *et al.,* 2017). Hemosiderin is an iron pigment in the ferric form (Fe+++) and is a byproduct of erythrocyte and hemoglobin breakdown (Leknes, 2015). Additionally, hemosiderin plays an important role in the production of antibacterial compounds, particularly hydrogen peroxide (Steinel and Bolnick, 2017). The presence of hemosiderin in the MMCs can be associated with phagocytosis of biological iron derived from erythrocytes' hemoglobin. Recent reports revealed that turtle MMCs can erythrophagocytose in vitro. The presence of destroyed erythrocytes and hemosiderin suggests that MMCs function in iron recycling, much like the hemosiderin-laden found in mammals. Turtle MMCs is described as "aggressively phagocytic," attacking bacteria, fungi, and helminth parasite eggs in vitro (Klei et al, 2017; Steinel and Bolnick, 2017). So, we can suggest that the carnivore diet of the Desert Monitor lizard may explain the significant increase of hemosiderin content in hepatic MMCs when compared with both Ornate Dabb lizard and Egyptian which are herbivorous.

ii. Melanin:

Melanin was evident in hepatic MMCs from all animals with varying prevalence (Table 1**).** In Ornate Dabb Lizard, Melanin was abundant in MMCs with highly HMB-45 immunoreactivity. HMB-45 positive cells grouped in nests or clusters in an organoid fashion, however, a single cell can predominate which indicates the presence of small free MMCs scattered in the hepatic parenchyma which were not demonstrated by H&E stain. Thus, this study can recommend the HMB-45 immunohistochemical stain as an accurate stain for MMCs demonstration. Hepatic MMCs of Egyptian Dabb lizard also revealed strong immunoreactivity to HMB-45, with a significant decrease when compared with those of Ornate Dabb lizard ($P < 0.01$), due to the difference in the size of MMCs, but the density of HMB-45 in each individual MMC is very strong. Another considerable difference between MMCs in Ornate and Egyptian Dabb lizards is the absence of free single MMCs in Egyptian Dabb lizards. Regarding hepatic MMCs in Desert Monitor Lizard, mild HMB-45 immunoreactivity was recorded as all MMCs appeared as pale aggregations that were rarely stained. Instead, most melanin-positive MMCs displayed yellowish or even almost no distinguishable color in HMB-45-stained sections (Fig. 6, Table 1).

The presence of melanin in MMCs is well documented (Akiyoshi et al., 2015; An and Zhang, 2019, Manrique et al., 2019). Normally melanin is produced by melanocytes developed embryologically from the neural crest and enveloped with

spherical to oval melanosomes. Melanin granules within MMCs were remarkably similar to those ones in the skin which indicates that the melanin of MMCs was derived from phagocytosis of melanosomes produced by classically occurring melanin-containing cells (Agius and Roberts, 2003). Melanin is composed of complex polymers that play a significant role inside the MMCs as a hydrogen peroxide neutralizer released in the fatty acid catabolism of cell membranes following phagocytosis. On the other hand, it has been proposed that melanin is either produced exogenously or internally by cells. This hypothesis is based on the ability of melanin to absorb and neutralize free radicals and other potentially toxic cations created from the phagocytic degradation of cellular material, allowing the body more time to respond to acute processes. Melanin may play a role in the synthesis of bactericidal substances, particularly hydrogen peroxide (Manrique *et al.,* 2019). According to various studies, hepatic hemocatherosis, hypoxia, and the activation of hepatic melanogenesis are all correlated (Akiyoshi *et al.,* 2015; An and Zhang, 2019). Indeed, Ribeiro *et al.* (2011) reported that activation of melanin regulates immunological reactions via the deposition of C3 protein components of the complement system, resulting in inflammatory responses. Okazaki *et al.,* (2015) suggested that MMCs may contain mainly pheomelanin, which has a yellow to reddish color so it can be rarely recognized as a pigment in H&E-stained section, whereas eumelanin can be easily detected as it is brown or black in H&E. However, limited reports about melanin types found in MMCs are available. High-performance liquid chromatography (HPLC) is the most accurate method for the quantification of pheomelanin and eumelanin (Kottler *et al.,* 2015).

5- Macrophages Lineage (CD68):

Comparing hepatic MMCs in each Ornate Dabb lizard, Egyptian Dabb lizard and Desert Monitor lizard showed the highest CD68-immunoreactivity and a significant increase ($P < 0.01$) of CD68 (%) stained area and macrophage Lineage in hepatic MMCs in Ornate Dabb lizard when compared with other two animals, as MMCs appeared as dense homogenous masses in dark brown color with highly CD68-immunoreactivity. Hepatic MMCs in Egyptian Dabb lizard showed moderate CD68-immunohistochemical reaction and MMCs appeared in light brown granular masses which indicates moderate CD68 stained area (%) stained area and Lineage of macrophages. In Desert Monitor lizard, hepatic MMCs showed the weakest CD68-immunoreactivity ($P < 0.01$) as MMCs appeared as small light brown aggregations. However, dark brown MMCs were rarely recorded (Figs 7, Table 1).

Macrophage accumulation in hepatic tissues of non-mammalian vertebrates especially reptiles was well documented (Akiyoshi *et al.,* 2015; Sales *et al,* 2017; An and Zhang, 2019, Manrique *et al.,* 2019). Macrophages are multifunctional cells that play a key role in the immune response. They phagocytose and break down foreign antigens as well as produce cytokines and chemokines that promote inflammation. Moreover, macrophages produce different growth factors and signaling molecules, thus macrophages are involved in the regulation of inflammation, wound healing and tissue repair (Brochhausen *et al.,* 2017; Mokhtar and Abdel Hafez, 2021). Clinical investigations reported the correlation between MMCs with a variety of highly resistant intracellular parasites and bacteria (Mokhtar and Abdel Hafez, 2021).

CD68 (Cluster of Differentiation 68) is a protein highly expressed by cells in the monocyte lineage such as monocytic phagocytes and osteoclasts, as well as by circulating macrophages, and by tissue macrophages as Kupffer cells and microglia. CD68 is a general macrophage marker and monocytes (Tremble *et al.,* 2020; Mokhtar and Abdel Hafez, 2021). CD68 is a glycoprotein that belongs to the lamp (lysosomal-associated membrane protein) – family. CD68 plays a role in antigen processing and in the lysosomal membranes' protection against lysosomal hydrolases (Mokhtar and Abdel Hafez, 2021).

Brochhausen *et al.,* (2017) reported that all types of macrophages, without differentiating the various macrophage phenotypes, represented a positive reaction for CD68. In hematopoietic tissues of goldfish *Carassius auratus*, the gene expression of CD68 was found to be formed during the maturation of macrophages and monocytes (Barreda et al. 2004). Phagocytosis assays indicated similar phagocytosis rates between lizards and mice bone marrow-derived macrophages, and both lizards and mice macrophage preparations revealed increased CD68 expression (Londono *et al.*, 2020). CD68 can be helpful in identifying heavily pigmented melanomacrophages that may mimic a conjunctival melanocytoma (Herwig-Carl, *et al.*, 2019).

In the current work, HMB-45 (Monoclonal Mouse Anti-Human Melanosome antibodies, (Code M0634, Dako, Copenhagen, Denmark), and macrophage markers CD68 Ab-4 (mouse monoclonal antibody, Thermo Scientific, code MS-1808-S0) were used for immunohistochemical staining of melanin and macrophages, respectively. Some studies used mammalian antibodies for immunohistochemical staining of reptilian tissues. An and Zang (2019), used polyclonal rabbit anti -Aquaporin 1 and 2 (anti-AQP1, AQP2) as primary antibodies (sigma, dilution 1:500) in the gallbladder of Qinghai lizard. Immunohistochemical detection of acidic alpha-keratin (AE1) and basic alpha-keratin (AE3) were Immunohistochemically demonstrated in normal tissues; Epidermis, Oesophageal epithelium, Gastric mucosa, small intestine, Liver, Tracheal epithelium, Lung, Kidney and Salt gland from bearded dragons (Pogona vitticeps) and loggerhead sea turtles (Caretta caretta). The AE1 mouse monoclonal antibody (IgG 1 isotype) (#61804; Progen Biotechnik, Heidelberg, Germany) was applied at 0.002 mg/mL (dilution 1:500), while the AE3 antibody mouse monoclonal (IgG 1 isotype) (#61806; Progen Biotechnik, Heidelberg, Germany) was applied at 0.001 mg/mL (dilution 1:1000) (Orós et al. , 2018). The immuno-localization of the pituitary adenylate cyclase-activating polypeptide (PACAP) and its receptors PAC1, VPAC1, and VPAC2 was demonstrated in the lizard Podarcis sicula gastrointestinal and respiratory tissues. A rabbit anti-PACAP antibody (Phoenix Pharmaceuticals, Belmont, CA, USA) was diluted at 1:300, while anti-PAC1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted at 1:300, anti-VPAC1 (Santa Cruz Biotechnology) was diluted 1:300, and anti-VPAC2 (Santa Cruz Biotechnology) diluted 1:400 in normal goat serum (Valiante *et al.*, 2009).

In summary, our findings revealed significant differences between the histological structure and the quantitative assessments of MMCs in Desert Monitor lizard and each Ornate and Egyptian Dabb Lizards. MMCs in Desert Monitor lizard were fewer in number, smaller in size; with decreased melanin pigments contents and macrophages lineages as expressed by CD68(%), and increased hemosiderin contents, which may indicate considerable differences between the immune system of Desert Monitor lizard and each of Ornate and Egyptian Dabb Lizards.

However, we can suggest that this difference in the immune system among the three reptiles may be attributed to the feeding habit of the Desert Monitor lizard as an example of a carnivorous reptile, by comparing with each of the Ornate Dabb Lizards as examples of herbivorous reptiles. Our suggestion is in accordance with Zimmerman (2020) who reported that the four orders of reptiles (Squamata, Tuatara, Crocodilia, and Testudines) differ substantially in a wide range of characters, including size, habitat, and life history, making it challenging to study the reptilian immune system as a whole. Thus, the immune systems of the four orders of reptiles vary in terms of their structural components, evolutionary background, exposure to pathogens, and possibly a wide range of other factors.

Another hypothesis for this difference in the immune system may be attributed to some possible environmental stresses in the area from which the ornate Dabb lizards were

captured. The significant increase in MMCs perimeter in Ornate Dabb lizard by comparing with Egyptian Dabb lizard, which has the same feeding habit, may support this hypothesis. However, further histological studies on the Ornate Dabb lizards from different areas, as well as, ecological and environmental studies are all required for more confirmation of this hypothesis.

To the best of our knowledge, this is the first study that deals with the comparative histological studies on MMCs among herbivores and carnivore reptiles. Also, basic knowledge of the mechanisms and components of the reptilian immune system was lacking.

Despite the difficulties and challenges of studying the immune system of reptiles, we believe in the importance of those types of research, which we urgently need in order to be able to study environmental pollution, as well as to confront climate changes, which is of great interest globally, and here we present our study as a first step on the road for comparative studies that correlate histology and histochemistry, with immunology, evolution, ecology, physiology and molecular genetics.

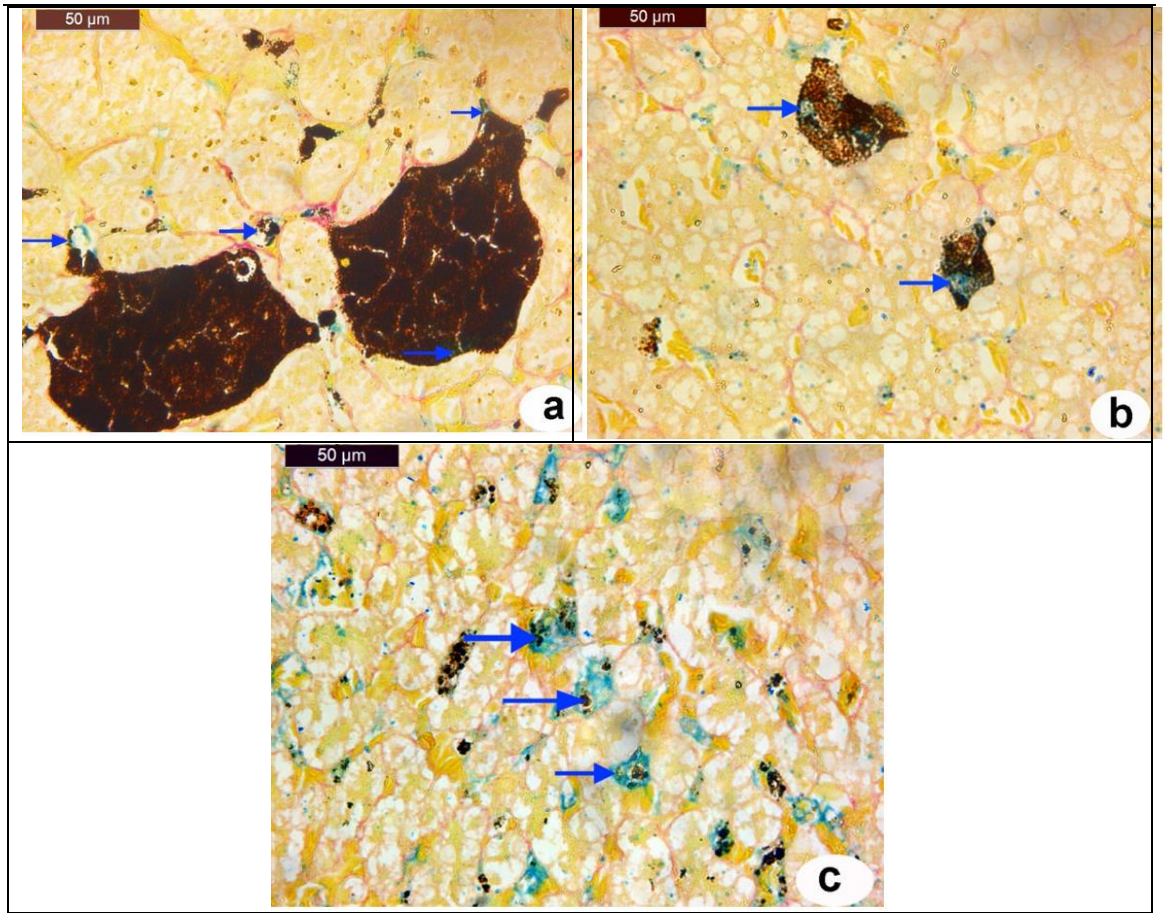

Fig. 5: Histochemical demonstration of hemosiderin pigments (blue color, arrows) in MMCs among the three reptiles (Perl's Prussian Blue; scale bar 50µm). (a) Section in the liver of Ornate Dabb lizard showing few traces of hemosiderin pigments within MMCs. (b) Section in the liver of Egyptian Dabb lizard showing the mild reaction of hemosiderin pigments within MMCs. (c) Section in the liver of Desert Monitor lizard showing large amounts of hemosiderin pigments within MMCs.

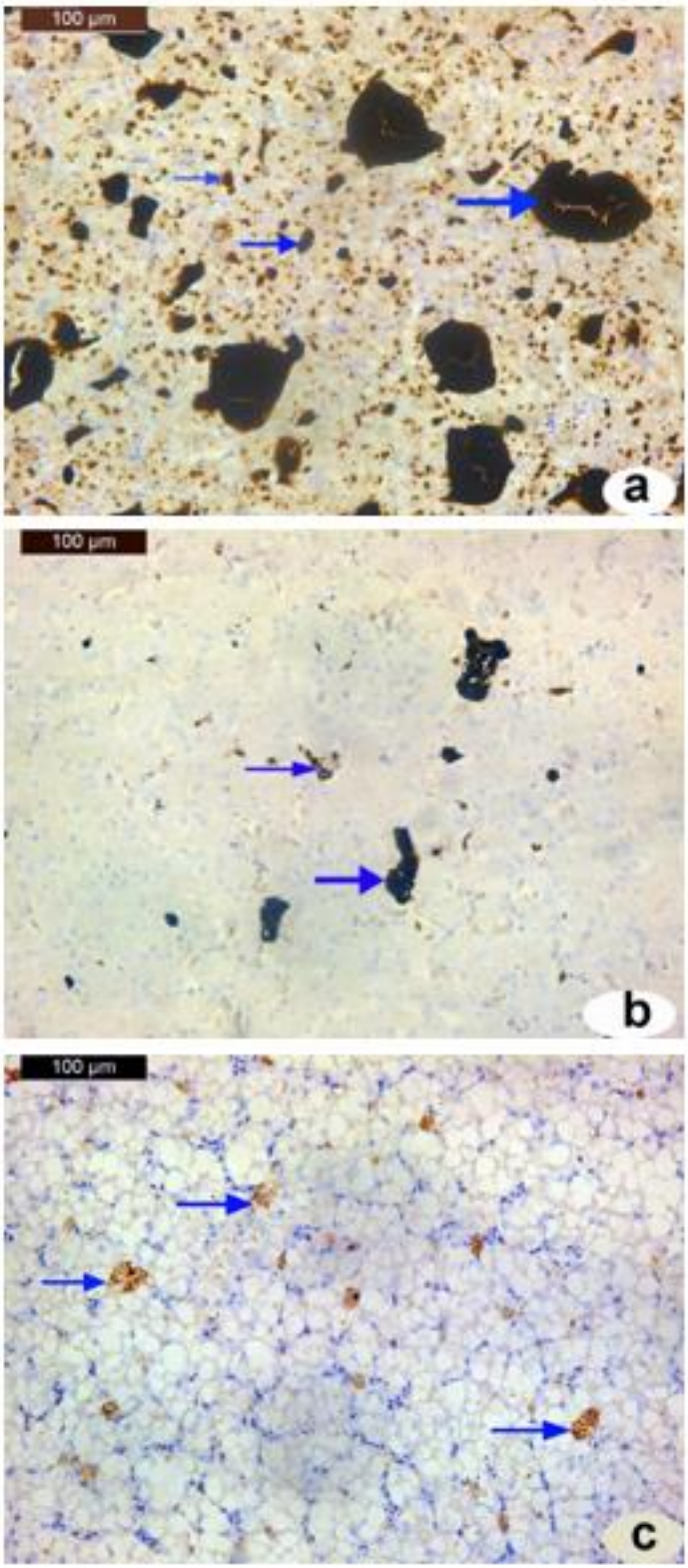

Figure (6): Immunohistochemical demonstration of melanin pigments (brown color, arrows) in MMCs among the three reptiles (HMB-45 immunostain; scale bar 100µm). (a) Section in the liver of Ornate Dabb lizard showing very strong immunoexpression of melanin pigments within MMCs. (b) Section in the liver of Egyptian Dabb lizard showing moderate immunoexpression of melanin pigments within MMCs. (c) Section in the liver of Desert Monitor lizard showing weak immunoexpression of melanin pigments within MMCs

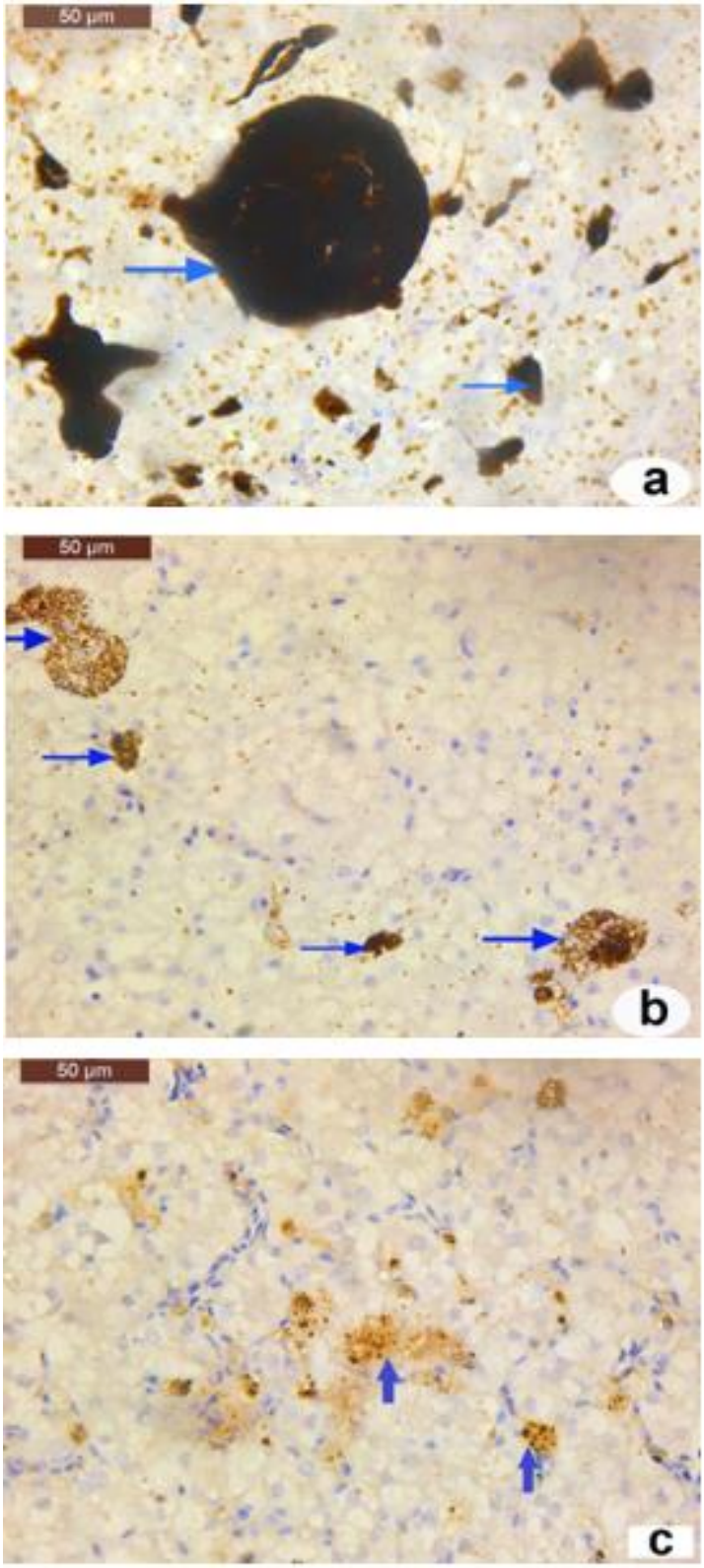

Fig. 7: Immunohistochemical demonstration of macrophage lineage (brown color, arrows) in MMCs among the three reptiles (CD68 immunostain; scale bar 50µm). (a) Section in the liver of Ornate Dabb lizard showing very strong immunoexpression of CD68 within MMCs. (b) Section in the liver of Egyptian Dabb lizard showing moderate immunoexpression of CD68 within MMCs. (c) Section in the liver of Desert Monitor lizard showing weak immunoexpression of CD68 within MMCs.

Conclussion

Our findings revealed significant differences between hepatic MMCs in Desert Monitor lizard and each of Ornate and Egyptian Dabb Lizards, in the histological structure and the quantitative assessments. In the Desert Monitor lizard, MMCs were fewer in number, and smaller in size; with decreased melanin pigments contents and macrophages lineages, and increased hemosiderin contents, which may indicate considerable differences between the immune system of the Desert Monitor lizard and each Ornate and Egyptian Dabb Lizards.

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Conflect of interest

No conflict of interest is associated with this work

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