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Disappeared Steps in Melanin Formation in *Octopus vulgaris* as a Model of Higher Organisms from the Red Sea, Egypt

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ABSTRACT

The melanin structure in Cephalopods comprises multi-compound structures, including Tyrosinase-A and Tyrosinase-B. These structures exhibit positivity for H&E stain in some stages and bromophenol blue stain in others. Other components are positive for H&E stains as amylase and peptides of holocrine comments, such as Gamma p.p.t and Gamma melodine, which are rich in ink of *Octopus vulgaris* and cause color blindness for prey during the predation process of animal. The *Octopus vulgaris* has numerous inking colors, such as brown and deep brown color, released during the escape from predation animals. During this process, the light falls on the skin, and melanin pigments give deep brown color by mixing the ink with amylase enzymes. The melanin mechanism in all organisms is similar to each other's in the animal kingdom except insects, where it is produced differently. Additionally, melanin pigments are only retained in the upper epidermis from bacterial reactions. The knowledge of the mechanism of melanin formations is highly important for treating dermatology diseases in humans and reducing the risk of skin cancer.

INTRODUCTION

Octopus vulgaris is a fast swimming, highly active predator and needs the inking process in many ways to escape from predation, concealments, changing color, secreting melanin to cause color blindness in its prey, and escaping from large predators **(Ye *et al.*, 2017; Abelreheem, 2022, 2023a, b)**. Some authors suggested that the inking process resembles the extraction of urine in humans **(Bames, 2001; Moreins *et al.*, 2012)**. Others mentioned that ink excretion during the animals' escaping from predation looks like urine excretion but with many complex toxic compounds. On the other hand, **Guinder-Parker *et al.* (2014)**, **Allouche *et al.* (2021)** and **Liang *et al.* (2023a, b)** elucidated that a small amount of ink acts as a narcotic for several animals' eyes, causing color blindness. All resemblance about the inking process has countless structures of bioactive compounds, if only taken in a natural environment. Some authors set forth the inking process from animals' resemblance to the holocrine excretion as an insulin metabolic process **(Allouche *et al.*, 2021; Chen *et al.*, 2021; Netcharoensirisuk *et al.*, 2021)**. The inking process contains true melanin pigment; this pigment is responsible for the skin color of humans, but with a small resemblance in structure in lower organisms. It imparts a deep brown color similar to that seen in humans. In octopuses with two stomachs, the formation of cartilage is observed **(Abdelrheem, 2022, 2023a, b)**. Some other invertebrate animals such as the butterfly (*Maskadomestica farobi*) have some bacterial from type *Kalabecraatean* sp. responsible for light production during the night, and, the squid from type *Galiteuthis* sp. produce the ink by the chemical reaction between amylase enzymes and some other components to produce the inking mixed with light produce bacteria. In *Octopus vulgaris*, this process is absent due to the presence of amylase and the absence of this type of bacteria. Thus, this study aimed to address and investigate all the disappeared stages of melanin formation by using various stainings. *Octopus vulgaris* resembles numerous animals with respect to the inking production such as squid and cuttlefish, and all are from the Octopodidae family, and they have different types of melanin, as proved by observations **(Derby, 2014; Jakop, 2015; Lili *et al.*, 2023; Shilpi, 2023)**. The inking process in this animal passes by multitudinous complex processes in the ink gland that are often misidentified, as the digestive gland appendix, a misnomer commonly used by cephalopod scientists. The Mollusca generally does not have the digestive gland appendix **(Frenzel, 1883, 1885, 1886)**; notably, the digestive gland is solely found in higher vertebrates. In invertebrates, the Siphonopoda as a higher organism has unique characteristics with regard to the bioaccumulation of heavy metals, with two true stomachs differentiated with complete enzyme secretions, and cartilage differentiated as human in addition to the melanin formation.

MATERIALS AND METHODS

Collection

Four samples of *Octopus vulgaris* were collected from two sites on the western coast of the Red Sea at the end of January during winter. The first site is 10km north of Al-Quseir City, while the 2^{nd} is located 17km south of Safaga (Fig. 1). Both collection sites have rocky shores. The samples were collected from the intertidal zone at the time of low tide. The collection was done by using the hand and packing it up. All specimens were collected and put in plastic containers containing seawater. Specimens were narcotic by adding menthol crystal, bought from El-Naser Chemicals Company in Egypt, to the water surface of the jar and waiting until relaxation. Specimens, dissected in the field to obtain the studied organs, were fixed and put in Blouin's solution for 24 hours for histological preparations.

Histological studies

Organs sectioned were cut off from the body and placed into Bouin’s solution in Sea water for 24 hours. Fixed parts were then passed to the graded series of alcohol from 100%. They were cleared in toluene three times each for 5 minutes then embedded in paraffin wax. Sectioning was made by microtome at 5- 7 µm thickness. Moreover, sections were stained with the following stains:

- Harris hematoxylin and eosin combination **(H&E) (Steedman, 1950**).
- For a demonstration of general proteins **(Mazia *et al.*, 1953**).

The slides were dehydrated through an ascending series of ethanol after staining. They were then passed through xylene to mounting medium and covered with coverslips.

RESULTS

Octopus vulgaris in the Red Sea looks like the ideal model in the world and has typical characters which are disrobed in many literatures (Fig.1).

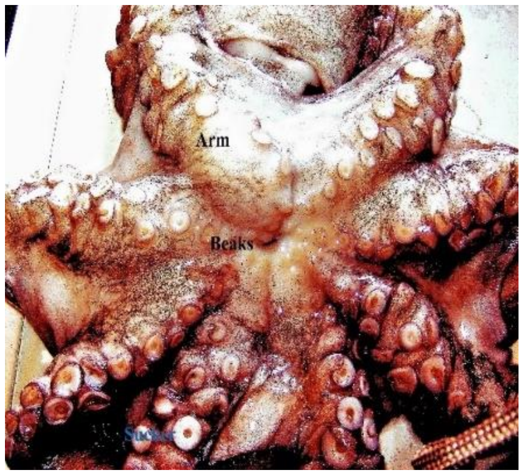
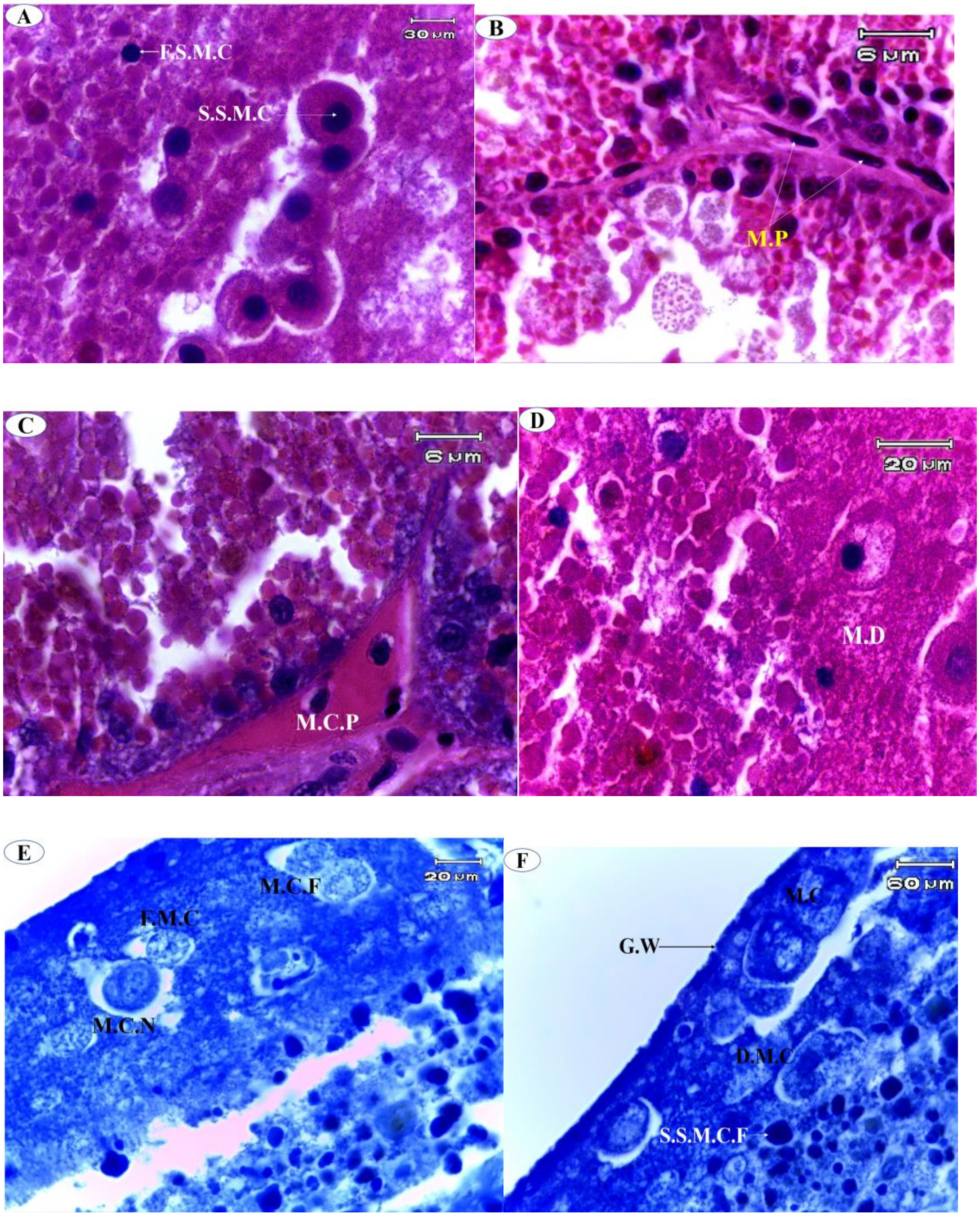


Fig. 1. The body plan of Octopus *vulgaris* (Cuvier, 1797). (Ventral view)

During dissecting the ink gland (a brown elongated palp form), ink was extruded from it, and the ink gland was obviously observed by the naked eye. The inking process includes some complex steps in animals such as amylase formation and Tyrosinase formation, but we can observe the rudimentary stages of the process in the natural field. The ink formation is divided into many processes, but it is impractical to observe each process individually. However, we can examine the different stages of inking in sections of the ink gland using different stains. Upon using the H&E stains, the sections were brought to light, and the ducts of the ink gland and the melanin that runs inside it were obviously remarked (Fig. 2A, B, C). The melanin particles are positive for H&E stains but the stages aren't positive due to the presence of Tyrosinase compound, which is a compound native by H&E stains. However, it is positive for bromophenol stains **(Barnes, 2001)**. By examining the sections by previous protein stains, we can take action to light the stages. The first stage is melanin formation, indicated by the presence of Tyrosinase compound, although they may not be visibly apparent, but the rudiment is apparent as the free cell from pigment (Fig. 2D). The second stage is the complete formation of the pigment; the cell is large and filled with melanin pigment (Fig. 2E). In the third stage, the cell is destroyed to extrude the pigment, and the remnant remains inside the ink gland (Fig. 2C). This stage becomes visible when stained by H&E due to the presence of pure melanin without Tyrosinase compound. However, we can observe in the next stage the presence of amylase enzymes by using protein stain. The fourth stage involves the diminishing of the cells containing pigment and reducing in size to pass through ink gland branches. The final stage involves releasing the ink containing melanin pigment (Fig. 2E, F, G, and H).



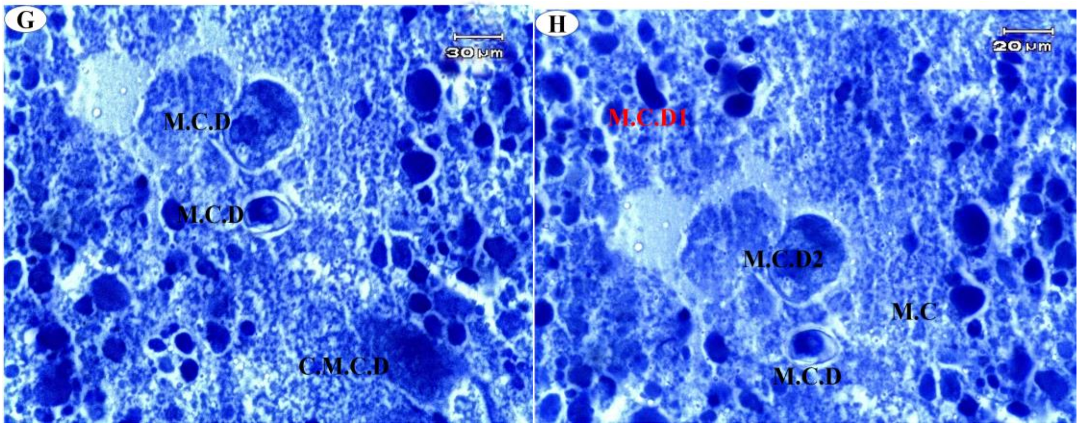


Fig. 2. Melanin formation stages in ink gland of *Octopus vulgaris* showing: **(A)** F. S. M.C: Formation stages of melanin cell, S.S.M.C: Second stage of melanin cells, **(B)** M.P: Melanin passing through ink gland branching, **(C)** M.C.P: Melanin passing, **(D)** M.D: Melanin destroyed, **(E)** M.C.F: Melanin cell free (Second stages), F.M.C: Free melanin cell (Third stages), M.C.N: Melanin cell nucleus destroy, **(F)** G.W: Gland wall, M.C: Melanin cell, D.M.C: Destroyed melanin cell, S.S.M.C.F: Second stages of melanin cell formation, **(F)** M.C.D: Melanin cell diminish in cell body, C.M.C.D: Cell Of melanin conclusion destroy, **(H)** M.C.D1: Melanin cell diminish in first step, M.C.D2: Melanin cell diminish in second step, M.C.D: Melanin cell diminish

DISCUSSION

Octopus vulgaris is similar to other invertebrates and has many defense ways to escape from predators. The assertion by authors such as **Ye *et al.* (2017)**, **Benito-Martinez *et al.* (2020, 2021)**, **Conntreras-Moreno *et al.* (2020)**, **Moreiras *et al.* (2021)** and **Lakhanova *et al.* (2022)** regarding the presence of an ink gland in octopus as a digestive gland appendix is based on incorrect investigations. It is scientifically inaccurate to consider the ink gland as part of the digestive gland appendix, as there is no true liver in siphonopods. The authors in this study suggested that the digestive gland appendix is the incorrect nomenclature in Mollusca since this term is absent from zoological scientists, Barfurth (1880, 1881, 1883), one of the earlier investigators, who described the digestive gland as a "hepatopancreas" and histologically established three cell types. In contrast, the ink gland in octopus is comprised of four distinct types of cells, making it more appropriate to refer to as multicellular **(Hao *et al.*, 2014)**. The inking process in octopus is complex and has complicated extrude compounds **(Moreiras *et al.*, 2021)**. The author's results come from the observations of other authors when describing the ink process formation; these observations describe the ink formation in the field of bioactive substances **(Sinha *et al.*, 2021)**. The findings of our study align with those of **Weinell *et al.* (2019)**, who suggested that the ink gland contains Tyrosinase and is positive for protein stain. The suggestion by **Moreiras *et al.* (2021)** that the ink gland contains melanin pigment is consistent with the findings of the present study. Furthermore, **Mustafa *et al.* (2010)** mentioned that the process takes place once a day during the diurnal; however that is the wrong emphasis.

The previous author failed to mention that predators can become hungry at any time during the predation process. Moreover, the author failed to observe the profound depths of the ocean and sea, where light is completely absent. Several authors **(Glavan *et al.*, 2015a, 2015b, 2019; Ye *et al.*, 2017; Yang *et al.*, 2018; Moreiras *et al.*, 2021; Reis *et al.*, 2022)** have suggested the excretion of melanin in certain animals, such as higher animals like humans, which contain amylase enzymes. These findings align with the results of the present study, which indicate the extrusion of melanin from melanin cells in all stages. The melanin travels from the ink gland to be stored in an ink sac, which naturally facilitates the excretion of melanin and ink.

Conclusion: This manuscript elucidates the mechanism of melanin formation in *Octopus vulgaris*, serving as a model for understanding similar processes in higher organisms. The author attempts to explains the mechanism as comprehensively and accurately as possible using suitable apparatus. This mechanism is key for addressing major dermatological diseases in medicine, and knowledge of it resolves many problems as vitiligo and certain types of cancer disease, which are associated with the absence of this pigment.

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