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Monitoring the Reproduction and Development of Eggs in the Sea Hare *Aplysia fasciata* under Laboratory Conditions

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Abstract: This study looks at spawning patterns, egg mass shape, and embryo development to learn more about the reproductive biology of the A. fasciata species that lives in Libya's coastal waters. In *June* 2022, eight mature *Aplysia fasciata* collected from Tajura coast and kept into aquarium contain aerated seawater and marine algae (*Ulva lactuca*). After five days, a mating couple was seen in the aquarium, and, a mass of fertilized eggs were produced. The clusters eggs were transfer to new aquarium. After few hours, the eggs started development to embryo and at 10 day the egg masses colour changed to a brownish as the embryo developed to the trochophore stage and started to rotate inside capsules. After 16 day of spawning the trochophore broke the egg capsules to hatch as free swimming larvae (the veliger stage). Unfortunately, after 25 day of fertilized eggs all larvae died as veliger. Probably, the result of inadequate conditions for rearing or lack an availability of appropriate food. Whereas *A. fasciata* has never been recorded before in the Libyan waters.

مراقبة تكاثر وتطور بيض أرنب البحر *Aplysia fasciata* في ظل ظروف المختبر

الكلمـــات المفتاحيـــة :
أرنب البحر *Aplysia fasciata*، التكاثر، يرقة التروكوفور، يرقة الفيلجر، ليبيا.

المستخلص: في يونيو 2022 ، جمعت ثمانية أفراد ناضجة من *Aplysia fasciata* من شاطئ تاجوراء، ووضعت في حوض يحتوي على ماء البحر، وغذيت بالطحالب البحرية الطازجة (*Ulva lactuca*) مع التهوية المستمرة. بعد خمسة أيام، شوهد زوج منها في حالة تزاوج ونتج عن ذلك كتلة من البيض المخصب. وتم نقل عناقيد البيض إلى حوض آخر. بعد ساعات قليلة من إنتاج البيض المخصب، بدأ البيض في التطور ليصبح جنينًا. بعد 10 أيام تغير لون كتل البيض إلى اللون البني مع تطور الجنين إلى مرحلة التروكوفور، وبدأ في الدوران داخل الكبسولات. كسرت التروكوفور كبسولات البيض بعد 16 يومًا من الإخصاب، لتفقس على شكل يرقات تسبح حرة (تسمى مرحلة الفيلجر). ولكن لسوء الحظ، بعد 25 يومًا ماتت جميع اليرقات على شكل يرقات فيلجر. ربما يكون ذلك نتيجة لظروف غير ملائمة للتربية، أو عدم توافر الغذاء. حيث لم يتم تسجيل *A. fasciata* من قبل في المياه الليبية.

INTRODUCTION

The sea hares, members of the Aplysidae family, are important grazers (Kajino et al., 2022). The genus Aplysia, often known as sea hares, is made up of 50 species that are found all over the world and graze primarily in the tidal and subtidal zones. Grouping under the following categories: Class Gastropoda, Subclass Heterobranchia, Infraclass Euthyneura, Subterclass Tectipleura, Order Aplysiida, Superfamily Aplysioidea, Family Aplysiidae, Genus Aplysia, and Species *Aplysia fasciata*. The benthic herbivorous Opisthobranchia, Aplysia, is found primarily in tropical and subtropical marine regions. (Beeman, 1968; Klussmann-Kolb, 2004; Lee et al., 2014). The *Aplysia* sp. it is a member of the Opisthobran-

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chia subclass, and the he reproduction, metamorphosis, growth, fecundity, and seasonal abundance of several aplysiid species have all been studied in various parts of the world (Kempf, 1981; Lee et al., 2011; Nimbs et al., 2017; Switzer-Dunlap & Hadfield, 1977; Yusa, 1996). Most gastropod exhibit gonochorism, and some clades as Opisthobranchia and Pulmonata are typically simultaneous hermaphrodites (Lee et al. 2014). The *Aplysia* sp. uses a variety of mechanisms, including inks, to avoid predators (Derby & Aggio, 2011). These organisms create eggs and sperm concurrently, but they often do not self-fertilize. Instead, they cross fertilize by copulating with another individual (Berry et al., 1994; Hadfield et al., 1984; Klussmann-Kolb, 2004; Kress & Schmekel, 1992; Painter et al., 1985; Plaut et al., 1995). The female role includes producing gelatinous egg masses in series and storing all sperm for transfer to another animal and the male is responsible for inducing the creation and transfer of aut sperm (Carefoot, 1987) and (Yusa, 1994).

In order to protect their fertilized eggs from severe environmental changes like salinity, temperature, desiccation, and water flow, many intertidal gastropod species encase their eggs in capsular egg masses (Lee et al., 2014; Przeslawski, 2004; Przeslawski & Benkendorff, 2005; Rawlings, 1999). Many marine invertebrate groups commonly encapsulate fertilized eggs, and the composition and shape of egg mass vary between species (Klussmann-Kolb & Wägele, 2001; Lee et al., 2014; Przeslawski, 2004; Przeslawski & Benkendorff, 2005) Egg capsules come in a variety of sizes and designs that range from millimeters to centimeters in length, from flat, hemispherical disks to towering, upright vases (Switzer-Dunlap & Hadfield, 1977).

Sea hare distribution in the Western Atlantic, which stretches from New Jersey to Brazil, and the Eastern Atlantic, which includes the Mediterranean Sea and the coast of West Africa (Valdés et al., 2013). Some individuals assume that *A. brasiliana*, a species found along the Atlantic coast of the Americas, is simply *A. fasciata* with a different regional colour pattern. Spread along the French Atlantic coast, in the Mediterranean, in West Africa, and on occasion, in the southern British Isles (Susswein et al., 1993). *Aplysia punctata* species found along the Catalan coast (NE Spain, Western Mediterranean Sea) exhibit unique color variants that differ in size, mating style, egg mass, and larval colour (Gonzalez et al., 2022).

Genus Aplysia's body colour is black and the parapodia, feet, and cephalic tentacles have a red border. The body occasionally has a few pale patches. The parapodial lobes are considerably apart from one another both anteriorly and laterally. Can grow to be 40 centimetres long (Bebbington & Hughes, 1973; Beeman, 1968; Klussmann-Kolb, 2004). The reproduction, metamorphosis, growth, fecundity, and seasonal abundance of several aplysiid species have all been studied in various parts of the world (Kempf, 1981; Kriegstein et al., 1974; Lee et al., 2011; Yusa, 1996). However, little is known about the reproductive traits and embryogenesis within egg masses of *A. fasciata*.

This study looks at spawning patterns, egg mass shape, and embryo development to learn more about the reproductive biology of the *A. fasciata* species that lives in Libya's coastal waters. One of the least researched species of Libyan fauna as *Aplysia* sp. There are reports of the spotted sea hare *A. dactylomela* in four different places in the maritime borders of Libya (Rizgalla et al., 2019). Whereas *A. fasciata* has never been recorded before in the Libyan waters.

MATERIALS AND METHODS

Adults of *A. fasciata* specimens were collected alive on June, 2022 from Tajura shore at Marine Research Centre, east of Tripoli, Libya (32°.89'61.31"N 13°.34'95.66"E) and transferred in seawater tanksto laboratory. Eight adult of *A. fasciata* were incubated in aquarium under control conditions (salinity

37 ppt ±1, pH 7.8, water temperature 21°C ±1, and aeration). Water of the aquaria was changed daily. *A. fasciata* were fed daily with fresh greenalgae (*Ulva lactuca*) based on (Gonzalez et al., 2022).

After five days, a mating was seen between couple of *A. fasciata* in the aquarium and, a mass of fertilized eggs was produced. The Egg clusters were removed from old aquariums to new one under the same condition as above (Ghory et al., 2020). After the eggs hatched, the larvae removed and placed in 1L flasks contain filtered seawater and microalgae (*Dunaliella salina*) as food. Leica binocular microscopes (Leica Microscope MZ9.5 With Tilting Binocular Head and Dual Illuminated Stand) and a digital camera (an Olympus TG4 compact camera and it is an automatic stacking feature). Were used to track the development and hatching eggs every day.

RESULTS

The body of *A. fasciata* was black colour, and the foot are very soft and flabby. Head and neck Wide and short. The well developed, flat, leaf-like cephalic tentacles. Tiny and distinct eyes; Foot is thin, thick, squishy, but hardens upon contraction, with well-defined edges, rounded front edges, and a short, blunt tail (Fig.1 A and B). A secretion from the ink gland that is dark purple, *A. fasciata* feeding on macroalgae of other surfaces. Egg masses appear as a long (15 cm) and yellowish masses, with jelly capsules (Fig.2 A and B). Morphological feature of eggs after fertilized were longs as noodle (Fig.1 F). Each capsule carried 18 to 25 egg, and there were 60 capsules per one centimetre (Fig.2 B and C). The masses were bright yellow after spawning (Fig.3 A). And this colour remained the same until embryos reached trochophore stage (Fig.3 B).

A. fasciata produced fertilized eggs through internal fertilization through copulation (Fig.1 C and D). After spawning, the egg masses were yellow in colour (Fig. 1), but as they developed, their colour changed (Fig.2 and 3). Embryogenesis at spawning, the fertilized eggs were spherical and had a diameter of around 80 µm (Fig.2 A). There was an unequal spiral cleavage during the cell division. About 12 hour after spawning, the first cleavage and development of a compact 2 cell embryo occurred (Fig.2 E). The cleavage of the two blastomeres was not synchronized during the second cleavage (Fig.2 E). After spawning, it takes around 18 hour for a compact 3 cell embryo to form (Fig.2 F). The four little blastomeres were split from the animal pole of each large blastomere during the third cleavage, which happened spirally. The first quartet was called by these tiny blastomeres. After spawning, it takes around 24 hour for a compact 8 cell embryo to form, and takes around 6 hour more to reach a 16 cell embryo (Fig.2 G).

At 10^{th} day after spawning, the colour of the egg masses changed to a brownish colour as the embryos progressed from the trochophore stage to the veliger stage. The embryo reached the trochophore stage and started rotating inside the egg capsule (Fig.3 A and B), and the trochophore larva develops to the veliger stage than broke the egg capsule and hatched as free swimming larva (Fig.3 C). They entered the veliger stage three more days later. The veliger between 12 and 15 day after hatch develop the prevelum in larva than becomes the velum at 16-17 day (Fig.3 D). Hatching larva has shell foot stick out and the velum covered with cilia; and there is inside digestive gland surrounded by jelly matrix (Fig.3 E and F). After 25^{th} day sadly, all larva died as veliger perphases.

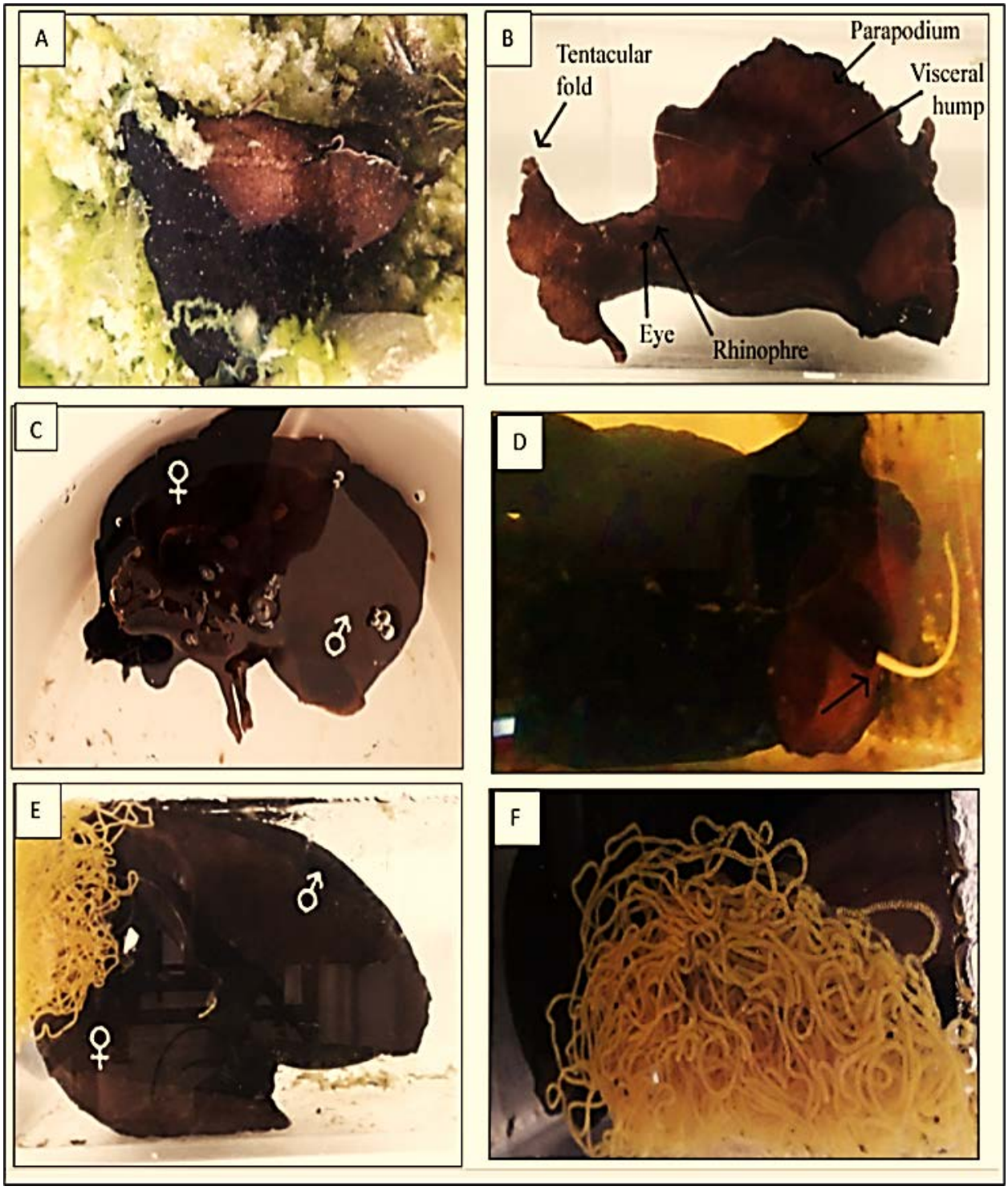

Fig (1). Morphological feature of *A. fasciata* (a, b), pair (male and female) (c), Penis of male (d), Egg masses after spawning (e) and masses of threads containing fertilized eggs (f).

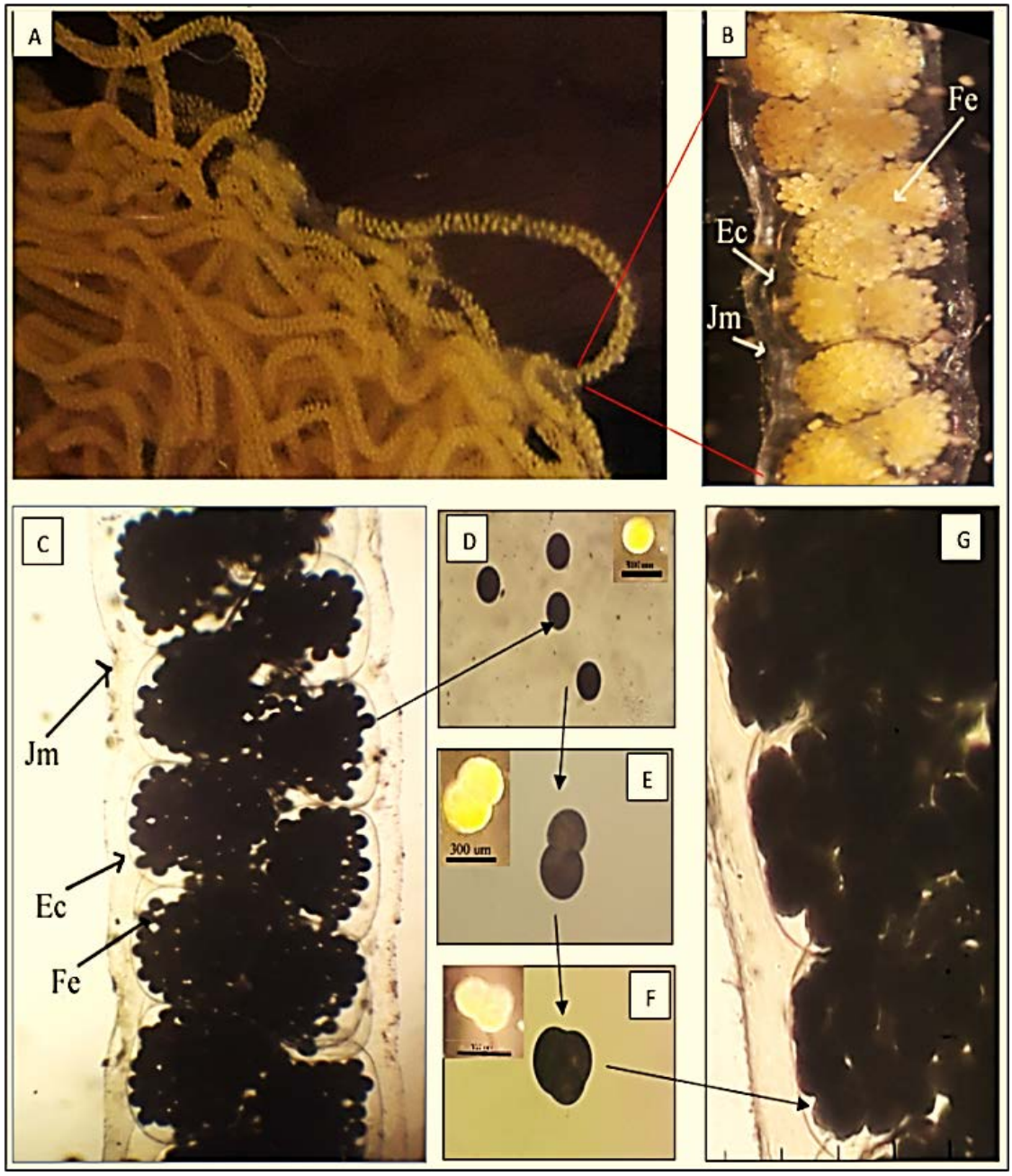

Fig. (2). Development of egg masses of *A. fasciata* in tank (A), formation of coupling chain and egg masses just after spawning (B), Change in colour of egg masses over developmental stage (C), Early developmental stage of *A. fasciata* fertilized egg (D), 2-cell stage embryo talks about 12hr after spawning (E), Beginning of 3 to 4 cell stage (F) and 8-cell and 16 cell ended with Trochophore stage (G). Scale bars indicate 300 µm. [Ec= Egg capsule, Jm= Jelly matrix and Fe = Fertilized egg / Scale bars indicate 10 cm].

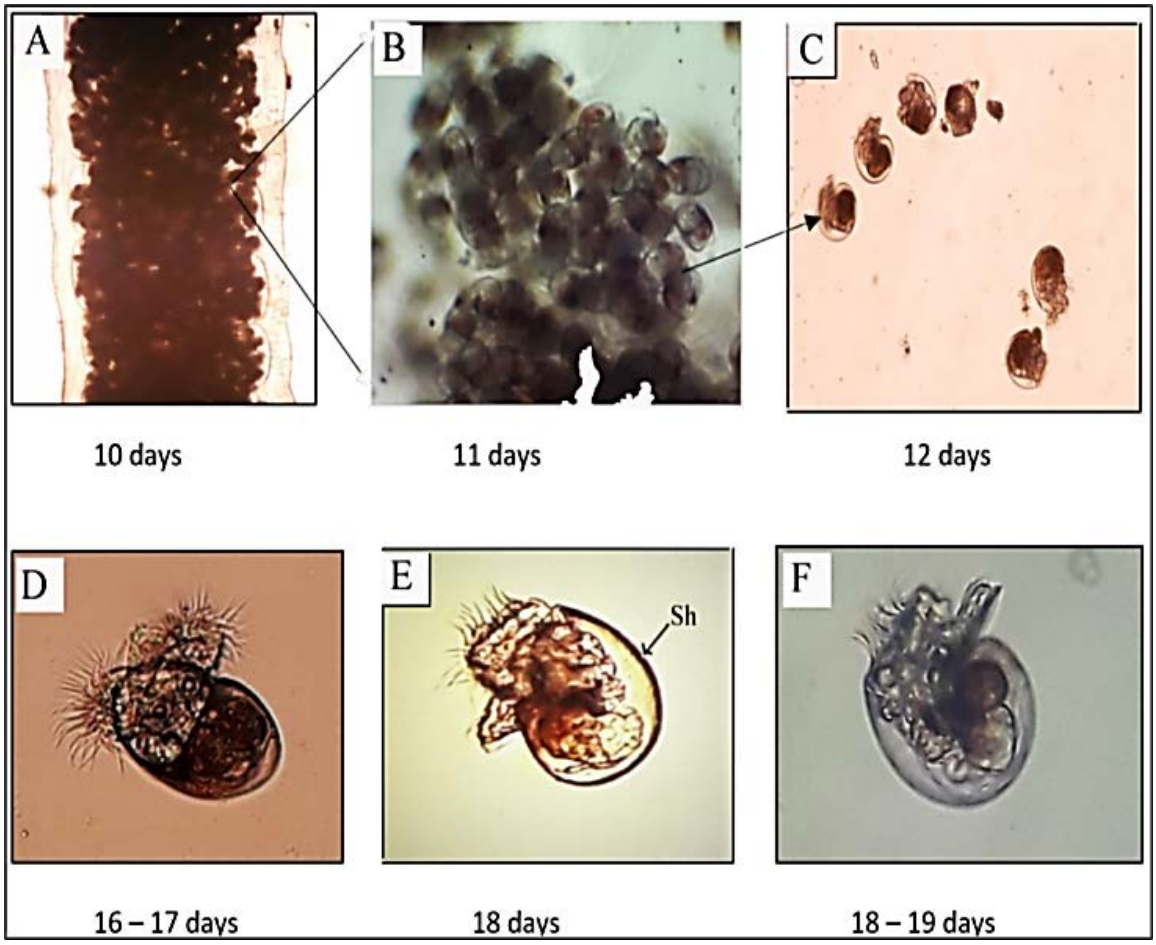

Fig. (3). Early developmental stage of *A. fasciata* (A), trochophore stage (B), veliger stage (C), and (D, E and F) hatching larva. [CI= cilia; FO= foot; JM= jelly matrix; LA= larva; PR= prevelum; SH= shell; VE= velum. Scale bars indicate 20 μm (A) and 500 μm (B to F).

DISCUSSION

The body of *A. fasciata* was black colour, and the foot are very soft and flabby and the Head and neck Wide and short. The well developed, flat, leaf-like cephalic tentacles. Tiny and distinct eyes; Foot is thin, thick, squishy, but hardens upon contraction, with well-defined edges, rounded front edges, and a short, blunt tail (Fig.1 A and B). A secretion from the ink gland that is dark purple. The first study of *A. fasciata* was made on March 12, 2022, when discovered adult animal of this species in the intertidal zone of the Tajura shore. This animal was kept in a laboratory with filtered seawater that was changed daily and was fed by green algae *U. lactuca* on a daily with continuously aerated. After a few days, spawned egg masses seen in the aquarium, *A. fasciata's* egg mass structure and egg development were observed under a light microscope, but no eggs were developing. Although the majority of opisthobranchia, including aplysiids, are simultaneous hermaphrodites, an adult animal has both a functioning female reproductive system and a functional male reproductive system. However, it produces egg masses through internal cross fertilization during copulation (Hadfield et al., 1984). According to the direction of facing, the varieties of copulation in opisthobranchia are either unilateral or reciprocal. In reciprocal copulation, two animals face one other in opposing directions; insert their penises into one another's common genital apertures, and exchange sperm (Eyster, 1986; Kandel, 1979; Lalli & Conover, 1973; Yusa, 1996), *Aplysia* spp. is the principal species that participate in unilat-

eral copulation. If there are more than two individuals present, a coupling chain is frequently created (Blankenship et al., 1983). This study *A. fasciata* participated in unilateral copulation with chain creation as part of it is mating behaviour. In chain copulation, the first animal assumed the role of a female while the second animal served as a sperm donor male to a female.

The characteristic of egg mass at spawning, the fertilized eggs were spherical and had a diameter of around 80 µm (Fig.1 F). The eggs embedded in jelly capsules that formed a cylindrical string (Fig.2 A and B). Each capsule carried 18 to 25 eggs, and there were 60 capsules per centimetre (Fig.2 B and C). The egg masses were a bright yellow colour after spawning (Fig.3 A). The colour of this egg mass remained the same until embryos development to trochophore stage (Fig.3 B). Many marine invertebrates commonly encapsulate their eggs within benthic egg capsules. Different opisthobranch has different egg masses structures and compositions. The Aplysiid's fertilized eggs are contained in capsules that are placed between layers of Mucopolysaccharide jelly to create a cylindrical string known as an egg masses (Przeslawski, 2004). The shape and structure of the egg mass of different Aplysiid species are very similar, but the proportion of capsules to egg mass and the quantity of eggs in each capsule differ between them. According to (Bridges, 1975 ; Lee et al., 2014; Switzer-Dunlap & Hadfield, 1977). There is an inverse link between size and egg count per capsule, however for many species with smaller eggs, the relationship is unclear. Additionally, the quantity of eggs per capsule increased with increasing animal body size as in *A. californica*, *A. brasiliana* (Capo et al., 2009).

At spawning, the spherical embryos and fertilized eggs had a diameter of around 80 µm (Fig.2 A). There was an irregular spiral cleavage during the cell division. The most of molluscs have been through spiral holoblastic cleavage, and egg size and temperature effect how an embryo develops. In opisthobranchia, the hatching size similarly rises with increasing embryonic time, and the egg diameter positively correlates with the size of the hatched veliger larvae (Hadfield et al., 1984). In a study of the growth of four Aplysiid species, it was shown that, at the same temperature, the embryonic periods vary between species with smaller and larger eggs, being shorter for the former and longer for the latter (Switzer-Dunlap & Hadfield, 1977). About 12 hour after spawning, the first cleavage and development of a compact 2 cell embryo occurred (Fig. 2E). The two blastomeres in the second cleavage did not split at the same time; the smaller blastomere split five hour after the development of the 2 cell embryo (Fig.2 E). After spawning, it takes around 18 hour for a compact 3 cell embryo to form (Fig.2 F). The four little blastomeres were split from the animal pole of each large blastomere during the third cleavage, which happened spirally. The first quartet was called by these little blastomeres. After spawning, it takes around 24 hour for a compact 8 cell embryo to form, and takes around 6 hour more to reach a 16 cell embryo (Fig.2 G).

A. fasciata larva produced fertilized eggs through internal fertilization through copulation (Fig.1 C and D). The mass of fertilized eggs was laid out in the shape of a string and were yellow colour (Fig. 1), but as they developed their colour changed to a brownish colour as the embryos development from the trochophore stage to the veliger stage (Fig.2 and 3). At 10^{th} day after spawning, the colour of the egg masses changed to a brownish colour as the embryos progressed from the trochophore stage to the veliger stage. The embryo reached the trochophore stage and started rotating inside the egg capsule (Fig.3 A and B), and the trochophore larva develops to the veliger stage than broke the egg capsule and hatched as free swimming larva (Fig.3 C). They entered the veliger stage three more days later. The veliger between 12 and 15 day after hatch develop the prevelum in larva than becomes the velum at 16-17 day (Fig.3 D).

Hatching larva has shell foot stick out and the velum covered with cilia; and there is inside digestive gland surrounded by jelly matrix (Fig.3 E and F). After 20th day sadly, all larva died as veliger per phase.

Although the particular time from egg laying to hatching varies between Aplysiid species, the range does not, it is typically around 16 days. The fertilized eggs of *A. fasciata* species underwent spiral cleavage, like those of other gastropod, but with unequal cell division. Within the range of other Aplysiids, the eggs hatched after 10 days spawning (Reverol et al., 2004). Unfortunately all larvae died as veliger stage, probably as a result of the unfavourable conditions of their rearing, such as contaminated water or lack of suitable food produce, as well as their longer developmental time to adult stage, suggesting that these stages may be especially sensitive to environmental changes.

CONCLUSION

Aplysia fasciata is one of the species, it has appeared recently in western coast of Libya and that has received less research. Therefore, this research focused on the spawning behaviour, number of eggs per capsule, egg masses structure, and egg growth and development. Moreover, the results of present study showed preliminary information about the reproductive of the *A. fasciata*, but unfortunately, all larvae died as veliger stage, possibly as a result of the unfavourable conditions in which they have been grown.

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ETHICS

There are no ethical issues of this manuscript.

Duality of interest: The authors declare that they have no duality of interest associated with this manuscript.

Author contributions: Abdulfattah Mohamed designed the experiment, applied it in the laboratory, and collected the data. Najla Mohamed followed up the experiment in the laboratory, analyzed the results, and wrote the manuscript.

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