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Effect of Bisphenol A (EDC) on the reproductive potential of *Helisoma duryi* (Wetherby, 1879)

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ABSTRACT

It has been claimed that bisphenol A (BPA) induces some reproductive potential disorders in the freshwater gastropods worldwide. The adult fecundity and egg hatchability as well as histological effects and embryonic deformities on the exotic freshwater snail *Helisoma duryi* have been studied to explore the superfeminization phenomena. The results of the 96h.LC_{50} of BPA for *H. duryi* was 4.06, and the histological examination for the hermaphrodite gland which was exposed to sub-lethal concentration showed considerable modification and changes in the semineferous tubules, number of eggs, spermatogenesis and the thickness of surrounding connective tissues. The results highlight the importance of obtaining basic knowledge of information on sub-lethal damage through the life-history of potential test species after exposure to test protocols.

Keywords: *Helisoma duryi,* Freshwater snail, Hermaphrodite, Egypt, BPA (Bisphinol A)

INTRODUCTION

An endocrine disrupter is one of the most exogenous substances that affects the endocrinesystem functions and consequently causesnegative effects on the process of hormone synthesis, secretion, transport, action, or elimination in an intact organism (US Environmental Protection Agency, 2004; 2007). Bisphenol A (BPA) is a widely used chemical in production of epoxy resins and polycarbonates, and is particularly abundant in PVC plastics (Vandenberg *et al*., 2009). The infant feeding bottles, milk, water and beverage bottles, processing equipment and water pipes are usually made of polycarbonates like BPA (Tsai, 2006). On the other hand, some of adhesives, building materials, powder paints, inner coating of cans, automotive lenses, compact discs, thermal paper, for encapsulation of electrical and electronic parts also are manufactured by BPA (Staples *et al*., 1998). The first use of BPA commercially was in 1905, and after three decades the first evidence of itsestrogenicity, that came from a conducted experiment on rats in the 1930s (Dodds & Lawson 1936, 1938) but it was not until 1997 that adverse effects of low-dose exposure on laboratory animals were first reported (Erickson, 2008). Gaido *et al*., (1997) confirmed the weak estrogenicity of BPA in vitro, showing it as about 15000 times less potent than 17 β-estradiol. Kuiper *et al*. (1997) demonstrated that BPA could interact with both the α- and β-estrogen receptors.

The major sources of BPA are environmental contamination and/or epoxy resin linings in the drinking-water distribution network (Bae *et al*., 2002). It should be noted that the BPA is oxidized during disinfection of drinking-water by chlorine, ozone and chlorine dioxide, leading to lower concentrations of BPA in tap water (Alum *et al.,* 2004; Lenz *et al*., 2004; Xu *et al*., 2007). Although most reported BPA concentrations in tap water are below 0.01 μg/l, some tap waters contained higher concentrations, up to 0.16 μg/l. Generally, BPA concentrations in water packaged in PC carboys (of 19 litres) are the highest values observed for drinking-water and are just below 1 μg/l; however, concentrations up to 9μg/l have been detected in four PC carboys (Biles *et al*., 1997; Cao & Corriveau, 2008c).

BPA are affecting the aquatic organisms such as snails, fish and insects when it released in aquatic environment as waste products. Snails are considered as a slow movable benthic fauna affected by large quantities of EDCs released in the environment. It likes many other invertebrates are poorly known because relationships between them are not clear, while their taxonomic inferences are often hindered by a lack of morphological diversification between different lineages, as occurs in different cryptic species or species showing overlapping variability (Elejalde *et al*., 2008).

The similarities or dissimilarities between some freshwater snails like *Marisa cornuarietes* or others were recently reported by Oehlmann and colleagues in their papers when they claimed that BPA induces superfeminization in the gastropod *Marisa cornuarietis*. These effects include formation of additional female organs, enlarged accessory sex glands, gross malformations of the pallial oviduct, and a stimulationof egg and egg mass production resulting in increased female mortality (Schulte-Oehlmann *et al*., 2001 Duft *et al*., 2003; Jobling *et al*., 2004; Oehlmann *et al*., 2000 and 2006a). By the same way, exposure to environmentally relevant BPA concentrations elevated fecundity in snails such as *Potamopyrgus antipodarum* and *Nucella lapillus* by increase of superfemales, characterized in females and reduction in sperm in males (Oehlmann *et al*., 2000) and development of snails such as hatchability of *Physa acuta* embryo (Argüello *et al*., 2012) like unshelled embryos (Duft *et al*., 2003). In some cases the adults of snails die after exposure to BPA for long run (Oehlmann *et al*., 2000 and Duft *et al*., 2003). However, measured BCFs in fish suggest that BPA has a low potential for bioaccumulation in aquatic vertebrates. A slightly higher potential is indicated for freshwater invertebrates (Heinonen *et al*., 2002). BPA is easily degraded (Howard, 1989), nevertheless it is regularly detected in aquatic ecosystems due to its continuous release into the environment, comparably to plasticizers such as phthalates (Oehlmann *et al*., 2008).

BPA concentrations of 12.5 mg/L stimulated larval development in *Taranis japonicus* (Oehlmann *et al*., 2009). In contrast, the copepod Acartia tonsa exhibited developmental inhibition at BPA concentrations above environmentally relevant levels (100 mg/L) (Andersen *et al*., 1999). Studies of reproductive effects due to BPA exposure have also been conducted for a variety of invertebrates (Flint *et al*., 2012). In the freshwater ramshorn snail (*Marisa cornuarietis*), exposure level >1.0 mg/L were found to result in superfeminization (additional female organs, enlarged sex glands, oviduct deformities, and increased fecundity), oviduct rupture, and mortality (Oehlmann *et al*., 2000). While developmental and reproductive effects in invertebrates have been reported due to BPA exposure, many were observed at levels currently well above environmentally relevant concentrations. However, there are few notable exceptions (Flint *et al*., 2012). The effect of BPA appears to vary considerably among related taxa, and it appears that some invertebrates may be hypersensitive to BPA exposure (freshwater mollusks, insect larvae and marine copepods in particular).

In the mollusc *Mytilus edulis*, spawning induction, as well as oocyte and ovarian follicle damage, was observed following BPA exposure for 3 weeks at 50 μg/L (Aarab *et al*., 2006). In the marine copepod *Acartia tonsa*, reduced egg production, reduced hatch success of offspring from exposed adults, and increased offspring mortality was found at exposures exceeding environmentally relevant concentrations

(>100 µg/L) (Andersen *et al*., 1999). Similarly, in the sea urchin *Paracentrotus lividus* a 30-min BPA exposure (300µg/L) reduced fertilization success approximately 42%, and increased larval deformities in the offspring of BPA exposed sperm (Arslan and Parlak, 2008).

While developmental and reproductive effects in invertebrates have been reported due to BPA exposure, many were observed at levels currently well above environmentally relevant concentrations. However there are a few notable exceptions (Flint *et al*., 2012). In Egypt, BPA research still scarce and need a lot of interesting researcher to investigate the numerous negative effects on the special tissues and on all the exposed animals. The previous work in this point of view in Egypt, simply summarized in some work of (ElShaer *et al*., 2013a, b; Sharf-El Deen *et al*., 2015). Hence, the aim of this study is to investigate the effect of BPA on one of the model animal of hermaphrodite freshwater snail *H. duryi* and the forms of superfeminization phenomena occurred.

MATERIALS AND METHODS

The Snails:

The freshwater snail (*Helisoma duryi*) was used in conducting the experiments in this study; were obtained from Abou-Rawash drainage area-Giza. The average width of *Helisoma duryi* was 14.85±0.59 mm and the high was 3.09±0.32 mm. manually sampling have been done during spring and summer seasons in (2012-2013). 492 collected individuals of *Helisoma duryi* were reared in the Laboratory of Invertebrate Biology (F_0), Animal House Building (AHB), belonging to Zoology Department, Faculty of Science, Al-Azhar University, Cairo. This collection of snails was maintained in well aerated aquaria filled with de-chlorinated water and fed on dried lettuce and fish feeding flacks (Tetramin). The third generation (F_3) of snails which reared in the laboratory was used to insure that they not exposed to any outside factors. Prior to experiments the snails were transferred to small tanks 3.5 liter capacity to acclimatize on space and other different control conditions. *Helisoma duryi* is an air breathing snail lives on freshwater plants as a herbivore and hermaphrodite snail of Planorbidae group.

Preparation of stock solution of BPA:

BPA stock solutions were prepared by placing a measured quantity of BPA (>99% pure) in a volumetric glass flask and bringing it to the appropriate volume with reagent grade or de-ionized water. The stock solutions were adjusted to approximately pH 11 by adding 5M sodium hydroxide (NaOH) in a drop wise manner. The resulting stock solutions were continuously stirred until the solutions contained no un-dissolved material. A stock solution was prepared every 7 to 10 days (Forbes *et al*., 2008).

Determination of 96h.LC_{50}:

The 96h.LC_{50} of BPA of *Helisoma duryi* snails were conducted according to Behreus and Karbeur (1953). Five groups of 10 snails were isolated in poly-containers (3liter), well aerated and filled with de-chlorinated water. A graduated algorithmic concentration of BPA was added to the treated containers in addition to the positive control in the rest container. The exposure was continued to 96h., of (0.25, 1, 5 and 10 ppt of BPA) plus ethyl alcohol which was added to the control group as a positive control. Daily observations for the treated groups were done to investigate the snail were dead or still a life. During the observation, the dead snails were removed immediately. The snail was considered dead (in the time of observation) by using dissecting needle and stimulating the foot or pushing the operculum in or out the snail. Sum of recorded dead snails after 96h., were used to calculate the value of LC_{50} according to the following formula:

$$LC_{50} = DM - \Sigma(z * d) / m$$

Where

DM = the higher dose used.
z = the number of dead snails of two successive doses divided by two.
d = the difference between two successive doses.
m = the number of snails in each group.

The main experiment:

The condition of chronic test was A 12:12-h light: dark photoperiod with two 30-min transition periods was used with illumination provided by fluorescent lights. Water temperature of cultures was maintained at 25^{o}C (Forbes *et al*., 2008). The experiments were designed to expose a certain number of snails to 10% from $LC_{50}$of BPA and collect samples on a regular basis for determination of histological studies. *Helisoma duryi* snails were divided into treated groups (300 snails), negative control group (100 snails) in de-chlorinated tap water and positive control group (100 snails) in de-chlorinated tap water with (NaOH). Each group was kept in a separate plastic jar (3.5 liter) fill completely by de-chlorinated tap water. The designated concentration (0.4ppt) of BPA was then applied to the treated groups. Then were samples collected from treated and control groups for histological studies every week.

Histological Studies:

In case of histological sample some treated and control(negative and positive) snails were dissected and cut the wanted portion of soft tissue which contain digestive gland and gonads then put in Bouan's solution for 24 hr. the samples of *H. duryi* fixed to maintains the soft tissue without damage, then removed to 70% ethanol until dehydration. All tissues were removed from 70% ethanol to path through sequence of ascending series of ethanol from 80%, 90%, 95% and absolute ethanol for 30 min in each concentration and duplicated in absolute ethanol. The dehydrated tissues were cleared in xylene: cedar wood oil (1:1, V / V) mixture for 24 h. or in Turbinol oil for 3 to 4 days then in xylene for 30 min before embedding, tell tissue be clear and transparent. The cleared tissues were pathed through xylene: Wax (1:1, V / V). Then the tissue was embedded for 4 replicates of wax for 15 minutes each inside the oven at 60°C,then blocked, and cutting by microtome of about 5-8 μ thickness. Deparafinized slides were stained with Mayer hematoxylin of Humason (1979) and Eosin 1% counter stain, Gomori trichrome stain of Gomori (1950) as well.

RESULTS

Determination of LC_{50}:

Figure (1) showed the changes in percentage of mortality for *Helisoma duryi* snails exposed to different concentrations of BPA for 96 h. The results showed that the calculated LC_{50} of BPA in case of *H. duryi* snails equal to 4.06 ppt. However, according to the regression relationship obtained by plotting the different concentrations against the percentage of mortality of snails, the relation between mortality and different concentrations was found to be significant according to the following equation:

% mortality = - 38.89 + (20.23 x concentration), with R_2 =0.83.

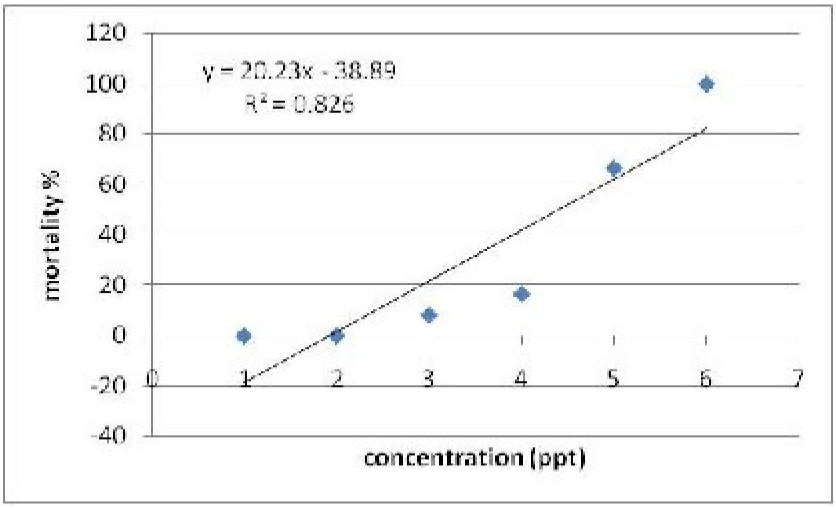

Fig. 1: The relationship between percentage of mortality and concentrations of BPA for *Helisoma duryi* snails.

Normal structure of hermaphrodite gland in Helisoma duryi:

Most of the aquatic gastropods are hermaphroditic animals which also depend on the cross fertilization between individuals of the same population. All there snails have the hermaphroditic gland and digestive gland complex at the apical part in the animal shell. The size of this gland differs according to age and maturity of the snail. The digestive gland-gonads complex occupies about 30-40 % of the soft parts of the small snails and increase in the adult once.

In transverse section of hermaphrodite gland of pulmonates, it consists of a matrix of flattened tubular glands (Plate I, A); each tubule contains enlarged head and long tail sperm which still inactive until fertilization and numerous in number and also it has small number of ova which have numerous food yolk surrounding the embryo.

The flattened tubules are lined outside by a thin layer of ebithelial cells consisting a basement membrane, which are separated from each other by thin layer of connective tissue (Plate I, A). Each tubular wall has numerous numbers of primary spermatocytes on the side opposite to the other tubular wall but at the free sides it has primary oocyte in small number (Plate I, B).

The histological effects of BPA on hermaphrodite gland in Helisoma duryi:

After exposing of *Helisoma duryi* snails to BPA for long period the stained sections of the hermaphrodite gland by Gomeri trichrome stain showed an increase in connective tissue sbetween the semineferous tubules and an increase in number of eggs at the expense of the number of sperms (Plate I, C, D). After one week of exposure the egg numbers were raising gradually with a slight increase of the surrounding connective tissue (Plate I, E). During the second week of exposure to BPA, the matured and pre-matured eggs increased in numbersand the basement membrane has been ruptured in some semineferous tubules and partially disappeared, in addition to dwindle in the number of sperm. After long period of exposure the vaculation appeared and the connective tissue became a thick layer between tubules and the tubules walls were destroyed and ruptured (Plate I, E, F).

Similarly, after exposure to BPA the spermatogenesis decreased and the spermatids also have been separated far away from the basement membrane and the sperms decreased in number as well. Sperm showed sporadic packages as a general view at the second week of exposure and still decreased in number (Plate I, G, H). After the long period of exposure sperm and spermatids disappeared and enlargement of lumen was observed. The effect of toxic material was observed at the end of exposure by full occupied tubules with different sizes of ova with semi-empty tubule of healthy sperms (Plate I, H).

On the other hand, by analyzing of data in the gonadal section we found that the number of tubules in the treated individuals of *H. duryi,* compared to the control one was slightly increased in number. But this inconsiderable extra numbers of semineferous tubules were enlarged enough to involve numerous amount of newly stimulated egg formation (Fig. 2). Hence, the egg numbers in the control experiment were significantly lower than that of the treated one; obviously one fourth of the treated individuals.

Consequently, during the treatment some subsamples of control and treated snails were collected and by counting the number of eggs inside the control and treated individuals we found that, the average number of control snails eggs were slightly constant and the number of eggs occurrence inside the tubules were fluctuated according to the laying eggs time (Fig. 3). In contrast, the treated snails had huge amount of eggs inside its tubules which significantly increased than the control snails. These excess of eggs in the treated snails tubules after laying eggs still higher than those of the control one and the total percent of egg occurrence go to the highest values by the end of exposure (Fig. 3).

Effect of BPA on egg masses hatchability of Helisoma duryi:

The normal structure of egg mass of the *H. duryi* snails varies according to the season and the health of the snails. Also, the time of hatching fluctuates between 11 days in summer to 15 days in winter. During the course of the study of the effect of BPA on the hatchability of eggs it was noticed that certain changes occurred in the egg capsule and/or the embryo of *H. duryi*. These observations could be summarized as follows;

1- In some snails, several egg masses have a relatively small number of eggs and the majority was without any embryos (Fig. 1).

2- The embryos inside the exposed egg masses didn't have the same stage of development (i.e. more than one stage in the same egg mass), such findings are not normal for this species of snails (Fig. 1).

3- Zero day eggs which have healthy embryos after exposure to BPA, had darkening or change of color (Plate II, B), specially for embryos exposed during the early stages comparing with the control (Plate II, A).

4- In some embryos (certain stages of gastrulaion) several vacuoles or air bubbles were seen inside the mass of gastrula, if compared with the control. These vacuoles developed later to complete degeneration of the embryos (Plate II, C, D).

5- Comparing with the control (Plate II, E), some embryos in the third stage of development mostly exhibited a loss in movement and extending their body outside the undeveloped shell (Plate II, F).

6- Comparing with the control, some exposed embryos in the pre-hatching stage stopped their movement; also several air bubbles were observed inside the embryo body indicating the beginning of death stage leading to body of the embryo starting of decomposing the embryo body leaving an empty shell (Plate II, G, H).

DISCUSSION

It is now generally accepted that endocrine-disrupting chemicals (EDCs), including BPA, are at least partially responsible for disruption of reproduction and development in some wildlife populations (Vos *et al.*, 2000 Flint *et al.*, 2012). Invertebrates are frequently used as bioindicators in EDC studies both in situ and in the laboratory. Research suggests that some invertebrates appear to be quite sensitive to BPA, and effects have been documented at environmentally relevant concentrations (Oehlmann *et al.*, 2009). The BPA effects found include altered abnormal blood hormone levels, reduced fertility and fecundity, masculinization of females and feminization of males. Several studies have observed developmental effects in invertebrates at various exposure levels (Flint *et al*., 2012).

Our study is one of the typical rear studies dealing with the histological deformation in the hermaphrodite gland of *Helisoma duryi* for long term study. The results of histology revealed some activities of increasing of superfeminization like enlargement of lumen of semineferous tubule which being crowded by numerous number of maturated eggs. These results agreed with most of studies which investigate the effect of BPA on the gonads of snails. Jubling *et al*. (2003) and Duft *et al*. (2003) found the similar increase of female fecundity of *Potamopyrgus antipodarum* with different concentration of BPA, but Forbes (2007) showed no effect on the gonad tubules in his experiment with concentration larger than the previous studies.

The value of LC50 of *H. duryi* was higher than other mollusk species, that may be due to its tolerant or resistance to the effect of BPA. This result is in agreement with that of the freshwater ramshorn snail (*Marisa cornuarietis*); exposure level >1.0 mg/L were found to result in superfeminization. By the excess number of eggs in the lumen and the oviduct, it gives the possibility to the oviduct and lumen to rupture and slight amount of heamolemph escape through the shell opening and heamolysis which lead finally to mortality like observed with Oehlmann *et al*. (2000) as well. While developmental and reproductive effects in invertebrates have been reported due to BPA exposure, many were observed at levels currently well above environmentally relevant concentrations (Flint *et al*., 2012). However there are few notable exceptions. The effect of BPA appears to vary considerably among related taxa, and it appears that some invertebrates may be hypersensitive to BPA exposure (freshwater molluscs, insect larvae, and marine copepods in particular).

In the present work the results provided evidence that even the laid eggs was not protected from the disturbing effect of BPA. The investigation of the relation between the BPA concentration and the percentage of hatched eggs showed evidence of positively correlated relationship. Meanwhile, even the juveniles that managed to hatch were those exposed in the early stages of development. Whereas those and even eggs after gastrula stage was when exposed to BPA if present in their surrounding environment. However, some snail species eggs may resist the BPA exposure due to the nature of the protein casing slow down the penetration of the toxic compound into the eggs.

The result of egg hatchability was slightly similar to that of unpublished thesis of TBTO male mimic endocrine disruptor (Zeina, 2005); the embryos go through the same for steps from zygote to final pre-hatching snail. Once the zygote pass the gastrula stage it is possible to continue to reach the final stage of embryonic development, but if the egg mass exposed to the BPA concentration before gastrula stage it would be stop the developmental stages.

CONCLUSION

The sublethal concentration of BPA on the freshwater snail *Helisoma duryi* has a gradually devastating effect on the hermaphrodite gland in particular, likewise rest of the snail tissues as well. The snail can resist the hazards effect during the first week of exposure but this resistance to the continuous exposure of BPA makes shifting from resistive to resigning. The results revealed the extent of the problem that, the snails and its eggs exposed to very tiny concentrations of BPA which reflect a huge damage and numerous effects on the snails and its embryos. These effects could be one of the main sources of the scarcity of the species.

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Plate I: Showed the effect of BPA on the hermaphrodite gland of *Helisoma duyri*; A- Normal structure of hermaphrodite gland showing typical lumen crowded with normal sperms (Sp) and ova (O), B- Showing normal lumen and the ova and sperms lined on the basement membrane (BM), C- 1st. week treated gonads showing vaculation (V) and numerous egg number, D- 2nd. week treated gonads showing excess number of ova (O) and dirndl in the sperm (Sp), E- 3rd. week gonads complete occupied with ova (O), F- 3rd. week scattering of sperms (Sp) and rupture of basement membrane (BM), G- 4th. week increase in the interstitial connective tissues (CT) between lumens with few number of sperms (Sp) and des-lined of spermatids bundles, and H- end of exposure showing no sperms (Sp) with huge amount of interstitial connective tissues (CT). (Hematoxyline and eosin for A,B and G; Gomori trichrom for C, D, E, F and H)

Plate II: Microphotograph showing different stages of embryo development inside the eggs of *Helisoma duryi*. Normal embryos: stage 1 (A), gastrula stage (C), third stage (E), pre-hatching stage (G) and the same stages after exposed to BPA. (B, D, F and H).

Plate I

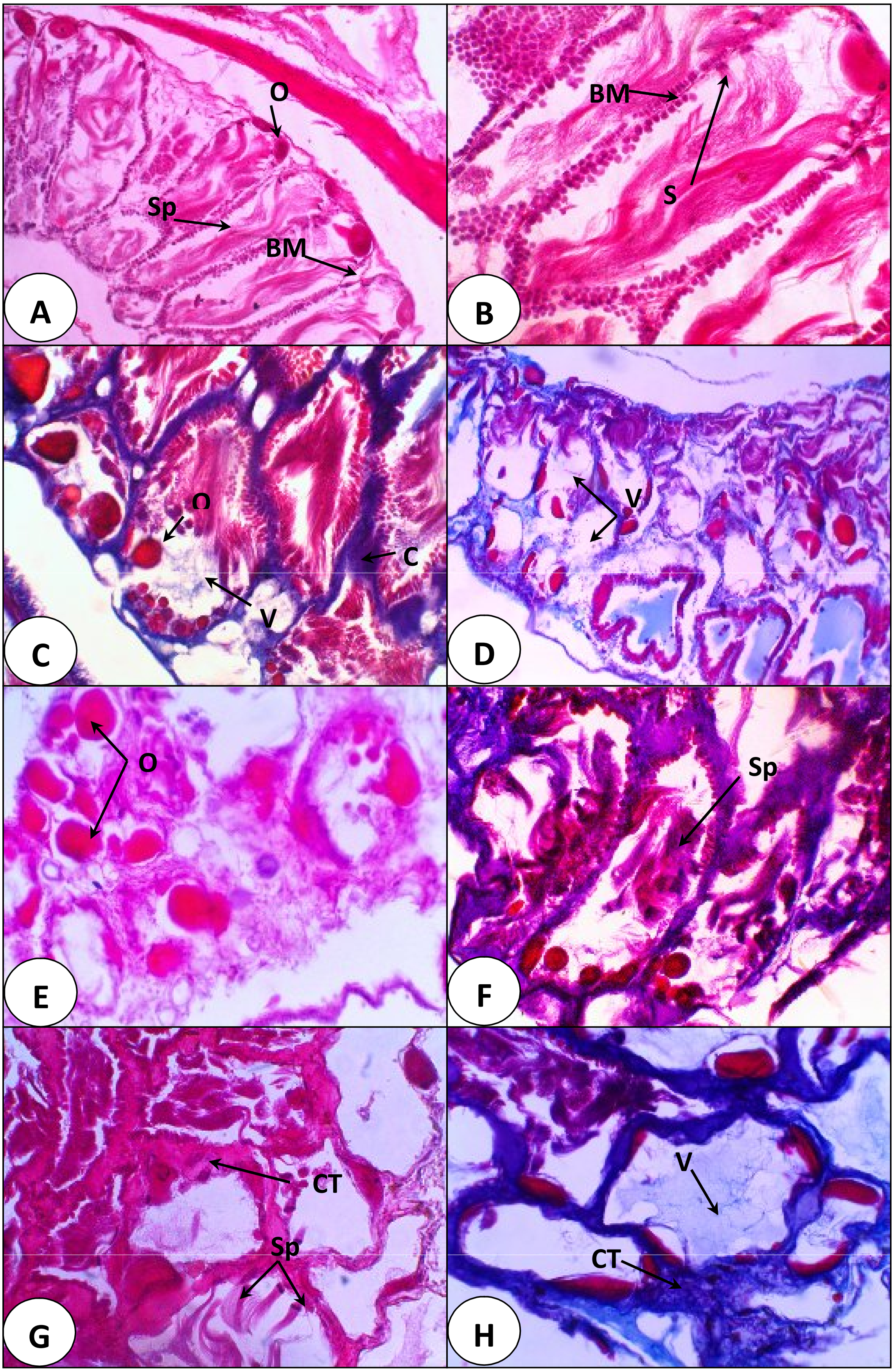
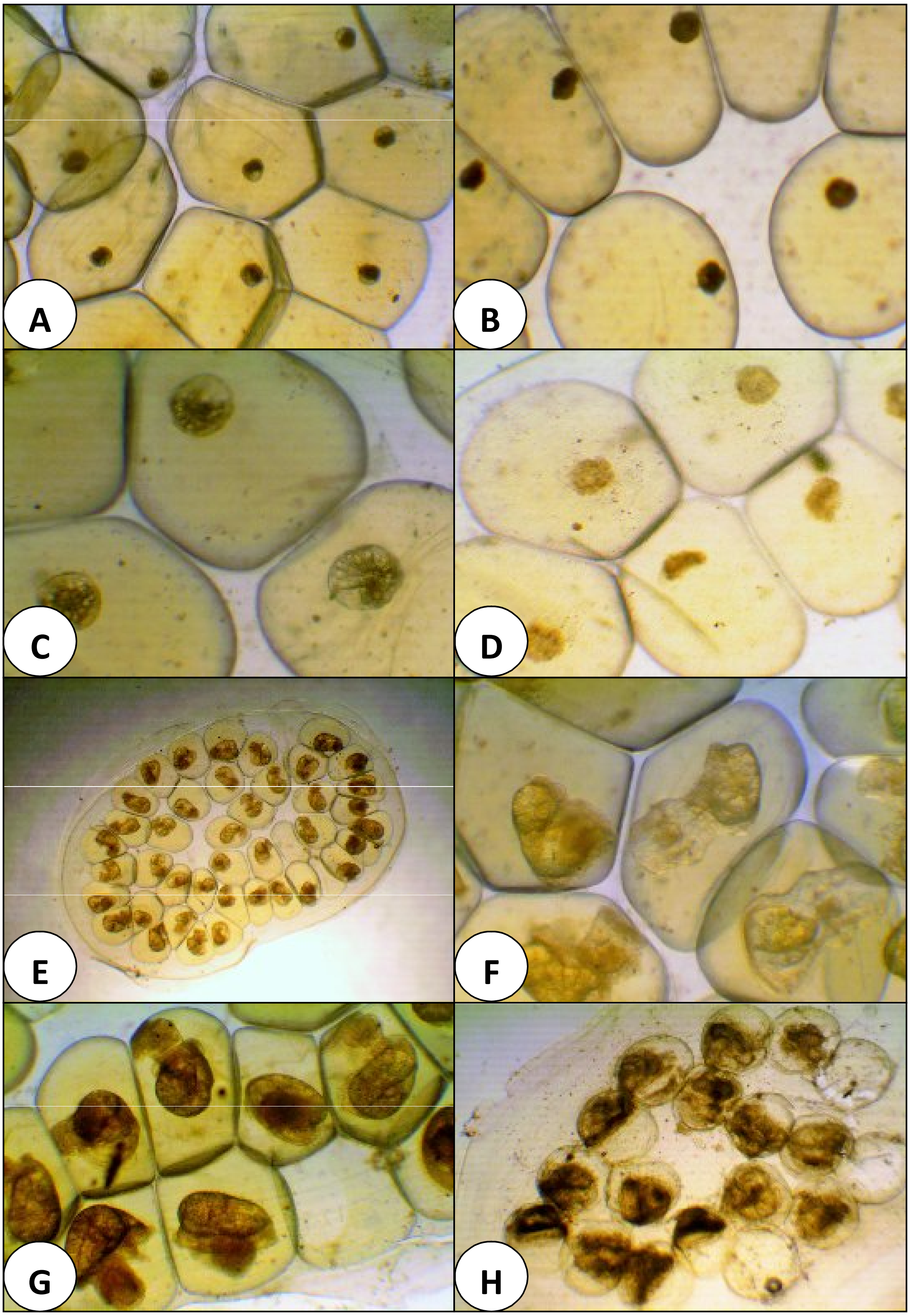


Plate II



ARABIC SUMMARY

تأثير بيسفينول – أ على القابليه الانجابيه علي قوقع (هيلوسوما ديوراى) ويسيربى، ١٨٧٩

مصطفى خالد جبر - عمرو فرج زينه - محمد محمود أبوزيد
شعبه علوم بحار واسماك ، قسم علم الحيوان ، كليه العلوم ، جامعه الازهر ، القاهرة ، مصر

لقد وجد ان بيسفينول – أ يحدث بعض الاضطرابات الانجابيه فى رخويات المياه العذبه فى جميع انحاء العالم. من خلال هذا العمل تم تحديد التركيز النصف المميت ل بيسفينوا – أ ودراسه تأثيره على ظاهره زياده الخصوبه الانثويه، خصوبه البالغين ,نسبه فقص البيض، التأثيرات النسيجيه والتشوهات الجنينيه على قوقع المياه العذبه (هيلوسوما ديوراى) داخل المعمل.أظهرتنتائج تجربه التأثير نصف المميت بعد ٩٦ ساعه لماده بيسفينول – أ لقوقع هيلوسوما ديوراى أنها ٤.٠٦، بينما اظهر الفحص النسيجى للغده الخنثويه للقوقع التى تعرضت للتركيز تحت المميت تأثيرا كبيرا وتغيرات فى القنوات المنويه وعدد البيض والخلايا المنتجه للمنى والانسجه الضامه المحيطه. والنتائج تسلط الضوء على اهميه معرفه الاساسيه للمعلومات عن التأثيرات تحت المميته من خلال تاريخ حياة الانواع بعد تعرضها لعده اختبارات وفحوص مختلفه.