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Endosulfan Exposure Reduced Fertilization, Hatching, and Survival Rate of the Lemon Fin Barb Hybrid Eggs and Larvae

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ABSTRACT

Endosulfan (ES) is an organochlorine insecticide that has been extensively used in agricultural production. Despite being banned globally, the production and illegal use of ES continue in certain countries, raising concerns about their impact on the environment and human health. The aim of this study was to examine the effect of ES on the fertilization, hatching, and survival rate of the lemon fin barb hybrid (LFBH; *Hypsibarbus wetmorei* × *Barbonymus schwanenfeldii*) eggs and larvae. A pair of LFBH was used as the broodstock. The sperm and eggs harvested via the stripping method were mixed and exposed to different concentrations of ES (0 ppm, or control; 0.01 ppm, 0.1 ppm, or 1 ppm). The fertilization and hatching rates were evaluated *in vitro* at 3 and 18 hours post-exposure, respectively. The survival rate of the larvae was assessed at 24, 48, and 72 hours post-hatching. The fertilization and hatching rates of the LFBH eggs treated with 1 ppm ES (44.24±4.6% and 18.54±2.8%, respectively) were significantly lower ($p<0.05$) than the control (63.35±5.8% and 46.76±1.3%, respectively). The main effect of treatment and time on the survival rate of the larvae was significant ($p < 0.019$) within three days post-hatchlings, where the survival rate of larvae exposed to 1 ppm was significantly lower ($p < 0.05$) than the control at every time interval. Overall, ES exposures displayed a detrimental effect on the early development and survival of the LFBH eggs and larvae.

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INTRODUCTION

Extensive agricultural activities demand the use of fertilizers and pesticides to ensure optimum yields and control of pests. Despite being highly regulated in many countries, the excessive use of pesticides remains a major issue of great concern. Studies have shown that less than 0.1% of pesticides applied reached the target pest, while the rest went into the environment, mainly into the water through leaching and runoff **(Pimental 1995; Pimentel and Burgess, 2012)**.

ES is an insecticide and acaricide that has been in use since the 1950s. Despite the benefits of controlling pests and mites, ES has negative impacts on the environment. Its persistence, toxicity, and ability to bioaccumulate contribute to these harmful effects **(Jayaraj *et al.*, 2016)**. ES takes many years to biodegrade, leading to contamination of environmental components such as soil, air, and water **(Sathishkumar *et al.*, 2021)**. This has raised concerns about its impact on the aquatic ecosystem, wildlife, and humans.

The incidence of ES toxicity in humans has been reported in various regions of the world **(Chugn *et al.*, 1998; Dewan *et al.*, 2004; Yavuz *et al.*, 2007; Kucuker *et al.*, 2008; Daglioglu *et al.*, 2011; Irshad and Joseph, 2015; James and Emmanuel, 2021)**. Acute ES toxicity in humans causes symptoms like nausea, vomiting, headache, dizziness, and convulsions, with severe cases leading to coma or death. Chronic exposure has been linked to neurotoxicity, infertility, developmental abnormalities, genotoxicity, and endocrine disruption **(Da Cuña *et al.*, 2016; Jang *et al.*, 2016; Zaman *et al.*, 2023; Priya *et al.*, 2024)**. In animals, ES poisoning has been documented in cattle, cats, dogs, and gaur **(Mor and Ozmen 2003; Sidhu *et al.*, 2006; Roma *et al.*, 2017; Radhakhrisnan, 2018)**. Due to its negative effects, ES has been classified as a dangerous pesticide and has been globally banned since 2012.

In Malaysia, despite the ban since 2005 **(Ramachandra *et al.*, 2006)**, illegal trade and use of ES remain, as evidenced by the detection of its residues in several rivers in the Malaysian Peninsula **(Leong *et al.*, 2007; Abdullah *et al.*, 2015; Haron *et al.*, 2015)**. This finding has been greatly associated with agricultural activities along the river systems **(Aminuddin *et al.*, 1996; Leong *et al.*, 2007; Haron *et al.*, 2015)**. Recently, ES and other contaminants, such as heavy metal residues and endocrine disruptors, have also been detected in water supply systems **(Wee *et al.*, 2021; Hasni *et al.*, 2023)**. This issue has become a public health concern and requires prompt action.

Major river basins in Malaysia provide a suitable environment for the breeding and growth of freshwater fish, thus boosting the aquaculture industry **(Weng and Mokhtar, 2010)**. However, deterioration of water quality may pose a challenge to this activity. The presence of pesticides, heavy metal residues, and endocrine disruptors can significantly impact freshwater fish. These substances have the potential to disrupt hormonal and reproductive functions, stunt growth, and delay the fish from reaching market size or weight. The productions of hybrid freshwater fish, which are highly resistant to environmental stresses and diseases, are poised to shape the future of aquaculture. In this present work, we have evaluated the effects of ES on the hatching and survival rates of the lemon fin barb hybrid (LFBH) eggs and larvae. This hybrid fish was developed in 2015 **(Zakaria, 2015)**, and to our knowledge this is the first study to use the LFBH hybrid for toxicity study.

MATERIALS AND METHODS

Animals

A pair of mature LFBH (*Barbonymus gonionotus* ♀ × *Hypsibarbus wetmorei* ♂), weighing approximately 250 g, were used as broodstock. The fish were obtained from the Fish Hatchery Centre at Universiti Putra Malaysia. All experimental protocols involving animals were approved by the Universiti Putra Malaysia's Animal Care and Use Committee (ACUC) (Ref no: UPM/IACUC/AUP-U006/2022).

Collection and Preparation of Gametes

The female and male fish were injected with 0.6 and 0.3 ml/kg Ovaprim (Syndel, Western Chemical Inc., MIF 900 - 001, Canada) respectively, prior to clutching, to induce final maturation. The eggs and sperms were gently clutched after 6 hours of injection.

ES Exposure

The eggs and sperms were gently mixed in a bowl using a chicken's feather, divided into four equal portions and exposed to either 0 ppm (control), 0.01 ppm, 0.1 ppm or 1 ppm concentration of ES. The mixtures were then transferred into a treatment tank (one tank/treatment), with the ambient temperature kept at 23 - 25 °C. A schematic overview of the experimental design is illustrated in **Fig.1.**

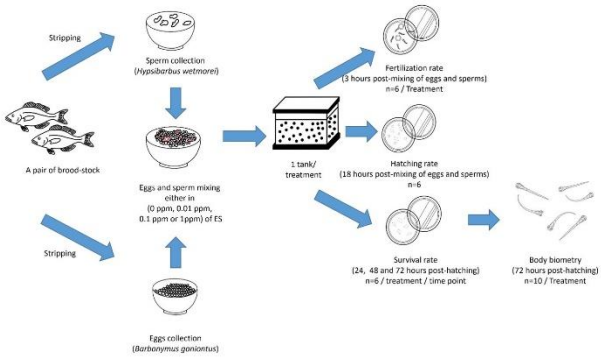

Fig. 1: Schematic overview of the study design. The eggs and sperm were obtained from a pair of broodstock via a stripping method: the male is of silver barb (*Barbonymus gonionotus*) and the female is of lemon fin barb (*Hypsibarbus wetmorei*). Eggs and sperm were mixed and kept in water composed of either 0 ppm (control), 0.01 ppm, 0.1 ppm, or 1 ppm of endosulfan (ES) and transferred to a pool tank (1 tank/treatment). The fertilization and hatching rate of the eggs were assessed and recorded at 3 and18 hours post-exposure, respectively. The survival rate was evaluated at 24-, 48-, and 72 hours post-exposure to ES. Body biometry of LFBH larvae was also taken 72 hours post-hatching (n=10/treatment) by measurement of the total length and the body depth.

Fertilization and Hatching Rate

The eggs from the treatment tank were randomly selected and transferred to a petri dish using a dropper. The fertilization and hatching rates were evaluated using 6 replicates per treatment (n=6). The eggs remained suspended in their respective treatment

solutions. The fertilization and hatching were examined under a stereomicroscope (Olympus 1.2 x 10) and Dyno-Eye (AM7025X), at 3- and 18 respectively, post-exposure to ES. The formulas for the calculation of fertilization and hatching rates are shown in **Figs 2a and 2b,** respectively.

Survival Rate

The survival rate of LFBH larvae was evaluated at 24, 48 and 72-hours post-hatching with 6 replicates per each treatment (*n*=6). Live larvae were observed and counted by the naked eye based on their motility. The formula for the calculation of the survival rate is shown in **Fig. 2c.**

(a) Fertilization rate (%): $\frac{\text{Number of fertilized eggs}}{\text{Total number of eggs}} \times 100$

(b) Hatching rate (%): $\frac{\text{Number of hatched larvae}}{\text{Total number of fertilized eggs}} \times 100$

(c) Survival rate (%): $\frac{\text{Number of live larvae}}{\text{Total number larvae}} \times 100$

Fig. 2: Formula for the calculation of (a) fertilization rate, (b) hatching rate of the Lemon Fin Barb Hybrid eggs, and the (c) survival rate of the hatchlings.

Body Biometric Evaluation

The body biometric of the larvae was measured and recorded at 72 hours post-hatching. Ten larvae (*n*=10/treatment) were randomly selected, and their total body length (TL) and body depth (BD) were measured and recorded with the aid of a stereomicroscope (Olympus 1.2 X) and Dyno-Eye (AM7025X, DinoCapture 2.0)**.** The TL was measured from the tip of the snout to the tip of the longest caudal fin lobe. The BD measured the maximum vertical distance between the dorsal and ventral margins of the larvae's body.

Statistical Analysis

All data are presented as means ± standard error of mean (S.E.M) and analyzed using one-way and two-way analysis of variance (ANOVA), as appropriate, followed by Tukey's HSD post hoc test. The level of significance was set at $P < 0.05$. Data were analyzed using the Statistical Package for Social Science (SPSS; Version 27, SPSS Inc., Chicago, IL, USA).

RESULTS

Fertilization and hatching rate

Both the fertilization and hatching rates demonstrated a declining trend over dosage (*i.e.*, control > 0.1 ppm > 0.1 ppm >1 ppm). The fertilization rate of eggs exposed to 1 ppm ES (44.24±4.6%) differed significantly ($p<0.05$; $P=0.041$) from the control (63.35±5.8%) **(Fig. 3a).** The hatching rate was significantly lower ($p<0.01$) in the group exposed to 1 ppm ES (18.54 ± 2.8%) compared to the control (46.76 ± 1.3%), the group exposed to 0.01 ppm ES (42.21 ± 5.6%), and 0.1 ppm ES (38.26 ± 8.4%) **(Fig. 3b).**

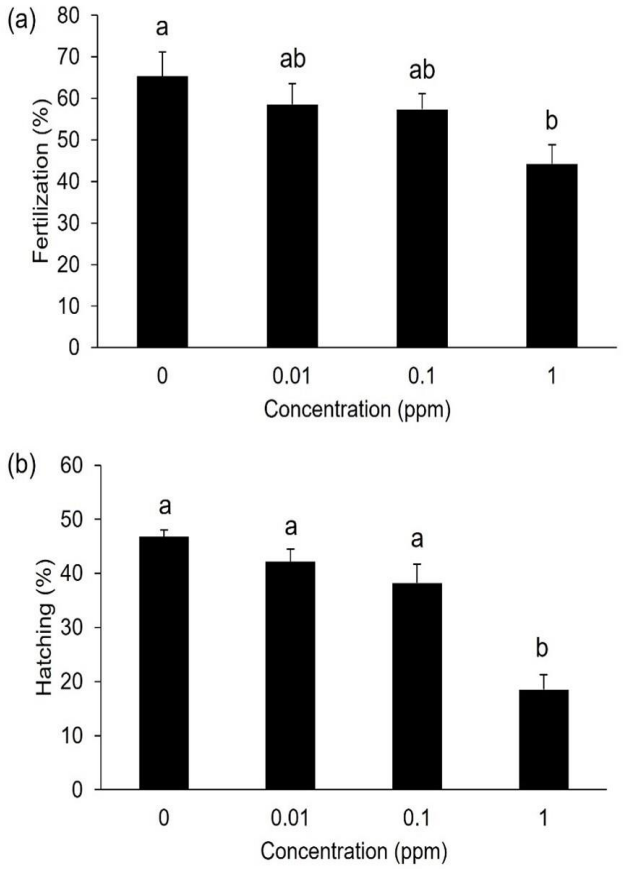

Fig. 3: Effects of endosulfan (ES) exposure on the (a) fertilization and (b) hatching rate of Lemon Fin Barb Hybrid (LFBH) eggs. The sperm and gametes were exposed to three different concentrations of ES, either at 0.01, 0.1, or 1 ppm. The control group was placed in an environment without ES (0 ppm). The fertilization and hatching rates were evaluated at 3- and 18 hours post-sperm and -egg mixing, respectively. Data are mean ± S.E.M. Data with different letters (a,b) differ significantly ($p < 0.05$).

Survival rate

There was a significant effect of treatment on the survival rate of larvae ($p < 0.05$; $p < 0.01$). The effect of time on the survival rate was also significant ($p < 0.05$; $p < 0.01$). The survival rate of LFBH larvae exposed to 1 ppm ES was significantly different from the control after 24 (92.20±1.9% *vs.* 82.98±7.7%), 48 (84.96±2.3% *vs.* 72.40±7.4%), and 72 hours (78.71±4.6% *vs.* 62.03±5.2%) **(Fig. 4).**

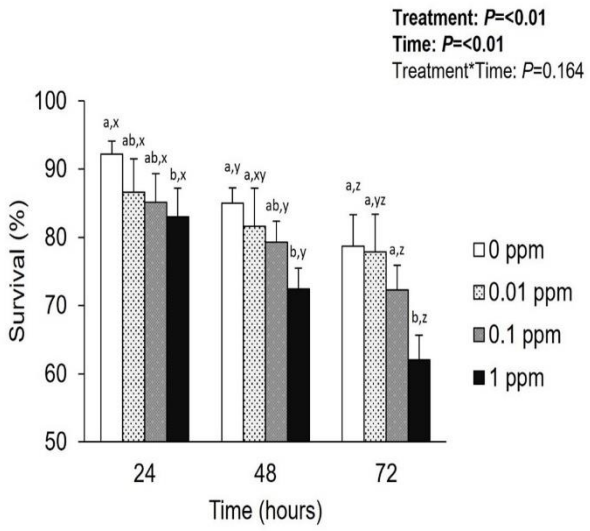

Fig. 4: Effects of endosulfan (ES) on the survival rate (%) of Lemon Fin Barb Hybrid (LFBH) eggs. The LFBH larvae were exposed to different concentrations of ES, either at 0.01, 0.1 or 1 ppm. The survival rate of LFBH larvae was evaluated at 24, 48 and 72 hours post-hatching. The control group was placed in an environment without endosulfan (0 ppm). Data are mean ± S.E.M. Data with different letters and differs significantly ($p < 0.05$) between treatment (ab) and over time (x,y,z).

Body biometric

There were no significant differences in the TL ($p > 0.05$; $p= 0.249$) and BD ($p > 0.05$; $p = 0.155$) of LFBH larvae exposed to different ES dosages at 72 hours post-hatching **(Fig. 5b and 5c,** respectively). In general, the TL and BD of LFBH larvae at 72 hours post-hatching were approximately 3.34±0.15 mm and 0.46±0.04 mm, respectively.

Embryo development

The fertilization and embryonic development of the fertilized LFBH eggs were observed over time **(Figs. 6a-f).** The unfertilized LFBH eggs were characterized by a cloudy appearance, disintegration, and sinking **(Fig. 3a).** Conversely, the fertilized eggs were evident by the presence of blastocyst and organogenesis **(Figs. 6b-f).** Fertilized eggs developed a spot (blastodisc) within 3 hours of fertilization (**Fig. 6b).** Organogenesis, where the head and tail end of the embryo were differentiated, occurred between 6 to 12 hours post-fertilization **(Figs. 6c-e).** The embryo was elongated and encircled the yolk materials at 12 hours post-fertilization **(Fig. 6e).** Finally, both tail and head ends were clearly defined by approximately 14 hours post-fertilization **(Figure 6f),** giving a complete appearance of the LFBH larva prior to hatching. No apparent difference in the morphology of the embryo was noticed, regardless of treatment.

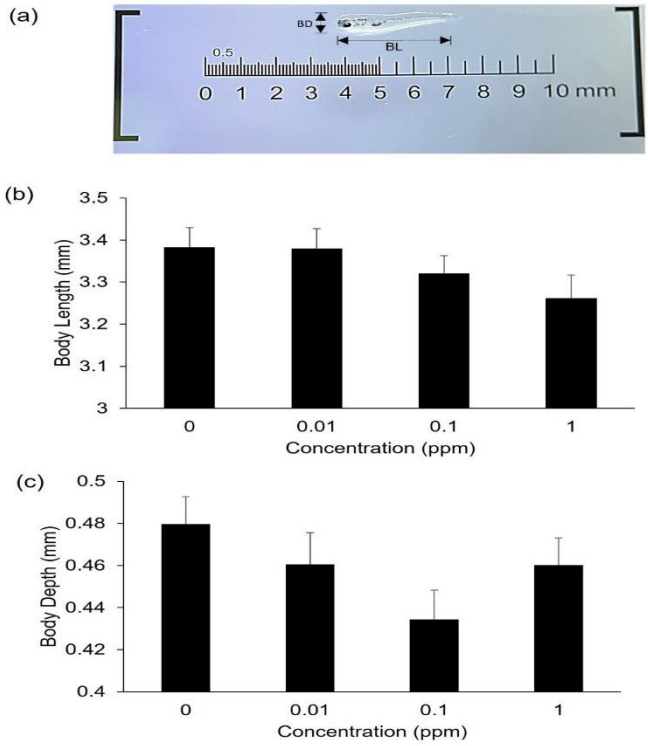

Fig. 5: Body biometry of Lemon Fin Barb Hybrid larvae 72 hours post-hatching. (a) Representative image of larvae observed under the stereomicroscope. Total body length (BL) length refers to measurements made from the tip of the snout to the tip of the longer lobe of the caudal fin. Meanwhile, the body depth (BD) indicated the measurement made from the maximum vertical distance between the dorsal and ventral margins of the larvae body. Bar graph showing the total body length (mm) and the (c) body depth (mm) of the larvae exposed to different concentrations of endosulfan, either at 0.01, 0.1 and 1 ppm. The control group was placed in an environment without endosulfan (0 ppm). Data are mean ± S.E.M. The data did not differ over dosage (p> 0.05).

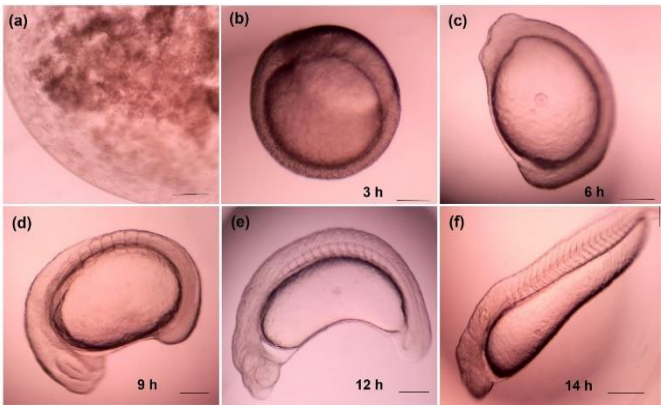

Fig. 6: The stages of embryonic development of the Lemon Fin Barb Hybrid (LFBH) over time. (a) Representative image of an unfertilized LFBH egg. (b-f) Representative images showing the fertilized LFBH eggs and developing LFBH embryo. (b) Late gastrula stage (c) Blastopore closure stage. (d) Embryo with somite and optic primordium, optic vesicle, olfactory placode and tail bud. (e) Advance stage showing the presence of the otic capsule, tail vesicle and lense formation. (f) The embryo became straight, losing the C shape, with the otolith appearance. (Scale bar: 200 µm).

DISCUSSION

LFBH is a crossbreed between *Hypsibarbus wetmorei* and *Borbonymus gonionotus* **(Zakaria, 2015; Zakaria *et al.*, 2018)**. This hybrid exhibits faster growth and better food conversion rates than its parent species **(Suharmili *et al.*, 2014; Sulaiman *et al.*, 2020)**. Generally, hybrid fish are known for superior traits, such as better survival rates, higher tolerance to low oxygen levels, and greater disease resistance **(Rahman *et al.*, 2018)**. Due to these advantages, LFBH could serve as a model for studying the toxicological effects of environmental pollutants on native Malaysian freshwater fish.

This study focuses on the effects of ES on the early development of LFBH. Our results showed that ES reduced the fertilization and hatching rate of LFBH. These effects were dose-dependent, with significant effects observed at 1 ppm exposure. Previous reports have highlighted the toxic effects of ES on aquatic life, including reproductive dysfunction, abnormal organ development, immune system impairment, and behavioural changes **(Chakravorty *et al.*, 1992; Chakrabarty *et al.*, 2012; Rajakumar *et al.*, 2012; Monde *et al.*, 2016; Hussein *et al.*, 2019)**. We believe that ES has damaged the structure and function of LFBH gametes, resulting in a reduced fertilization rate. One of the mechanisms is through the suppression of vitellogenesis in the oocytes **(Chakravorty *et al.*, 1992; Sukardi *et al.*, 2019)**. Vitellogenin, a female-specific reproductive protein, provides nutrients for developing embryos and aids in immune defense against pathogens **(Zhang *et al.*, 2015).** Another potential direct effect of ES on fish fertilization rates is the reduction in sperm motility, viability, and count **(Choudhary and Joshi, 2003; Balasubramani and Pandian, 2008; Rajakumar *et al.*, 2012)**. In mammalian sperm, ES has been shown to disrupt sperm integrity, cause chromatin decondensation, and induce DNA fragmentation. **(Sanchez *et al.*, 2018)**.

We found that ES reduced the survival rate of LFBH larvae over time. In addition to impaired movement, poor food uptake, and inefficient digestion, ES may also disrupt the osmoregulatory function of aquatic organisms. **(Bhavan, 2000; Altinok and Capkin, 2007; Bernabo *et al.*, 2010)**. Significant structural changes were observed in the gills of prawns exposed to ES **(Bhavan, 2000).** In trout, ES exposure caused lamellar oedema, separation of the lamellar epithelium, lamella fusion, and swelling of epithelial cells **(Altinok and Capkin, 2007)**. The gill is crucial for gas exchange, osmoregulation, ion balance, excretion, and hormone production **(Bhavan, 2000; Herrero *et al.*, 2018)**. Therefore, damage to gill structure and function can prevent fish from adapting to changes in salinity, ultimately leading to death **(Su *et al.*, 2002)**.

We initially expected ES exposure to negatively affect the physical growth of LFBH larvae. However, body biometry analysis showed no significant differences between treatments. This contrasts with findings in zebrafish and snakehead fish, where larval growth suppression was observed. **(Balasubramani and Pandian, 2008; Sharmila and Abhik, 2013)**. ES accumulated in zebrafish muscle, impairing swimming, foraging efficiency, and food digestion and absorption **(Sastry *et al.*, 1982; Balasubramani and Pandian, 2008; Parerira *et al.*, 2012)**. These effects of ES were not observed in our study, possibly due to the shorter exposure time. In our study, ES exposure lasted only 3 days post-hatching, whereas previous studies were conducted over 21 days or more.

In this present study, we also briefly monitored the embryonic development of LFBH. Our observations were consistent with the report by **Zakaria *et al.*, 2018)**, who also examined the embryonic development of the same hybrid in a normal environmental setting. We found that the LFBH reached complete organogenesis at approximately 14 hours post-fertilization. This appears to be faster than the rosy barb, which may take around 18 hours to reach the same stages **(Bhattacharya *et al.*, 2005).** Since LFBH carries half barb genetics, our findings suggest that the hybrid may tend to have a faster embryonic development rate than its parent stock. This is further supported by the fact that *Barbonymus goniotus* took approximately 15 hours to reach complete organogenesis and around 16 hours post-hatching **(Myint and Soe, 2020)**. Studies in zebrafish show that ES could disrupt the development of embryos **(Stanley *et al.*, 2009; Zaman *et al.*, 2023)**.

CONCLUSION

ES exposures reduce the fertilization, hatching and survival rate of LFBH eggs and larvae. These preliminary findings provide valuable insights for future studies on ES toxicity in other native freshwater fish in Malaysia.

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Conflict of interest

The authors declare no conflict of interest.

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