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Fertilization, hatching, and embryogenesis of diploid and triploid eggs of *Anabas testudineus* (Bloch, 1792)

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Summary

This study investigated the breeding parameters and embryogenic development of diploid and heat shock-induced triploid eggs of *Anabas testudineus* (Bloch, 1792). To this effect, broodstocks of *A. testudineus* were induced to spawn using the Ovaprim® hormone. After fertilization, the eggs were divided into two groups and one portion heat shocked at 41°C (for 3 min), at approximately 4 min after fertilization. Results of fertilization, hatchability, as well as the sequence and timing of embryogenic development were collated from three breeding trials. Fertilization percentages were similar in both treatments (≈90%) while hatchability was higher in the diploid eggs (79.56%) than the triploid induced eggs (50.04%). Both treatments had the same sequence of embryogenetic stages; however, the timing of development was significantly delayed in the triploids (i.e. beyond the 2-cell stages) as compared with the observations in the control group (diploid eggs). Consequently, hatching time was 5 h faster in the diploid eggs [i.e. 18 hours post fertilization (hpf)] compared with the triploid induced eggs (23 hpf). The most critical stage of embryonic development in which mass mortality occurred in the different treatments was the somite stage. The status of triploid hatchlings was affirmed using erythrocyte morphology in 2-month-old fingerlings.

Keywords: Climbing perch; Embryogenesis; Erythrocyte morphology; Heat shock; Spawning performance

Introduction

Triploidy induction in fish is not a new concept as it has been successfully demonstrated in several aquaculture species (Felip *et al*., 2001; Nell, 2002; Tiwary *et al*., 2004; Maxime, 2008, Piferrer *et al*., 2009). The popularity of this method over other chromosome manipulation techniques is largely due to the ease of producing sterile aquaculture progenies or progenies with reduced sexual functionality (Allen & Stanley, 1978; Brown & Roberts, 1982; Neal, 2003). Historically, scientific references on triploidization date back to the 1940s (Makino & Ozima, 1943; Svardson, 1945). Since then, many triploid aquaculture species have been commercially exploited. Hence, improved performance characteristics such as growth, meat production (Piferrer *et al*., 2009; Berrill *et al*., 2012; Fraser *et al*., 2012) and by extension economic benefit and effective large-scale production of sterile fishes have been widely exploited in commercial triploid fishes (Dunham, 2004; Maxime, 2008).

In addition, mass production of triploid fishes can be used to minimize genetic and ecological disorder that might result from interactions between wild and cultured fishes (Cotter *et al*., 2000). Triploid fishes are therefore not considered to be 'genetically modified organisms' (Piferrer *et al*., 2000; Maxime, 2008). Moreover, triploidy also occurs naturally in the wild (Thorgaard, 1983). The occurrence of triploidy in the

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wild is largely because of the occasional failure of the extrusion of the second polar body in fertilized eggs (Thorgaard & Gall, 1979) due to environmental changes or hybrid stabilization (Legatt & Iwama, 2003).

Under laboratory conditions, however, triploid fish are produced by inhibiting the release of this polar body II during the second stage of meiosis by shock treatment (Carman *et al.*, 1991; Piferrer *et al.*, 2009). It is well known that shock-induced eggs for triploidization lead to reduced hatchability (Piferrer *et al.*, 2000, 2003). The mystery behind this general observation in many previous studies could be unravelled by studying the embryogenetic development of the fish. However, this approach has not been given much attention in the past. Therefore, the effect of shock treatment on the timing of embryogenetic development is largely still unknown.

The non-pigmented and transparent nature of the *Anabas testudineus* (Bloch, 1792) egg, which facilitates easy observation, makes it one of the most important animal models for embryonic development studies in fish. In addition, this fish is a popular and highly priced aquaculture candidate in India, Malaysia, and several south-east Asian countries (Chaturvedi *et al.*, 2015). Hence, its performance and aquaculture value could be increased by inducing triploidization. The 'climbing perch' (as it is commonly called) are naturally domicile in the freshwaters, brackish waters and estuaries of several countries such as Pakistan, India, Nepal, Bangladesh, Sri Lanka, Southern China, Myanmar, Thailand, Singapore, Indonesia, Malaysia, Laos, Vietnam, Brunei and the Philippines (Talwar & Jhingran, 1991; Chondar, 1999; Jayaram, 2010; Pal & Chaudhry, 2010). This study is specifically aimed at determining the spawning and embryonic development of diploid and heat-shocked triploid eggs of *A. testudineus* under controlled laboratory conditions.

Materials and Methods

Sexually mature broodstocks of *A. testudineus* obtained from wild catches were maintained separately in fibreglass tanks at the School of Fisheries and Aquaculture Science hatchery of the Universiti Malaysia Terengganu, in Malaysia. The broodstocks were acclimatized for 1 month before being used for this study. During the period of acclimation, they were fed commercial feed [45% crude protein (CP)] to satiation twice daily. Breeding and embryogenetic development reports for this study are the cumulative observation from three different breeding trials using two pairs of male and female brood fish (per trial). Both the male and females were injected with Ovaprim® hormone at a dose of 0.5 ml kg^{-1} of the body weight. They were separated into different rearing tanks (80 × 60 × 40 cm^3) and allowed to swim freely for a latency period between 10 and 12 h. Eggs were then stripped from the females into a clean bowl by gently pressing the abdominal region of the fish. Similarly, gentle pressure was applied to the abdomen of the male broodstocks to extract the milt into a separate bowl. Fertilization was then done by mixing the eggs and milt as well as activation with water.

The fertilized eggs were quickly divided into two bowls as the treatment and the controlled group. The portions of eggs for the treatment group were 'heat shocked' at 41°C (for three min), to induce triploidization approximately 4 min after fertilization. However, the control group was not heat shocked. The control and treatment groups of eggs were immediately incubated in already prepared triplicates batches in a 100 litre fibreglass tank with continuous aeration. Water quality before and after heat shock (i.e. during the incubation) was monitored and kept optimum (temperature = 28.5 ± 0.4°C; pH = 7.13 ± 0.90; conductivity = 559 ± 3.88 µS cm^{-1}; total dissolved solid = 254.0 ± 0.91 mg l^{-1}; and dissolved oxygen = 5.06 ± 0.38 mg l^{-1}). It is important to note, that due to the temperature sensitivity of studies such as these, digitally regulated heaters set at 28°C were used to maintain the temperature of the triplicated treatments baths throughout the incubation period/embryogenesis studies.

About 50 eggs were collected from each group (in Petri dishes) and observed under a Nikon dissecting microscope (Model number C-DSLS). A new batch of eggs was obtained for observation on 10-min basis through the early cleavages stages and on an hourly basis at the advanced stages of embryonic development. The different developmental stages were captured using a Sony camera (Cyber-shot 16.2MP Model number: DSC-TX10 50i) fitted to the microscope while the time of attainment of each stage was recorded as observed with a stopwatch. This monitoring was continued from the point of fertilization up to when the fry was hatched. It is also important to note that incubation in the triplicate fibreglass tank and

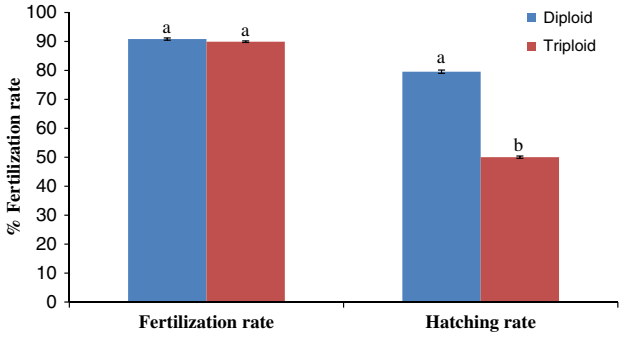

Figure 1 Spawning performance of the eggs of diploid and heat shock-induced triploid *Anabas testudineus*.

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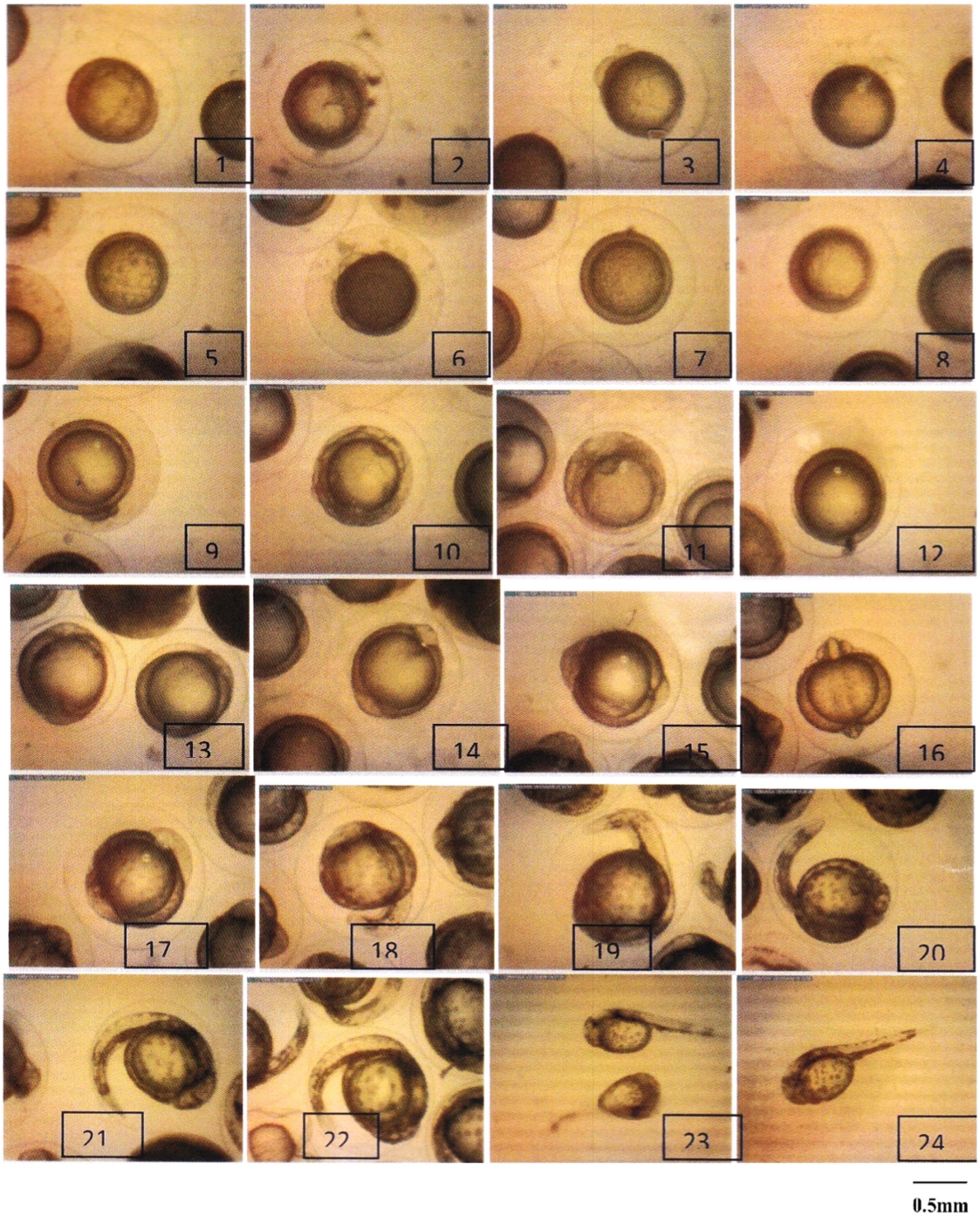

Figure 2 Normal embryogenesis as observed in the diploid or triploid developing eggs of *Anabas testudineus* under laboratory conditions. (1) Blastodisc formation. (2) 2-cell stage. (3) 4-cell stage. (4) 8-cell stage. (5) 16-cell stage. (6) 32-cell stage. (7) 64-cell stage. (8) 128-cell stage. (9) Morula stage. (10) Early blastula stage. (11) Mid-blastula stage. (12) Late blastula stage. (13) Pre-early gastrula stage. (14) Early gastrula stage. (15) Mid-gastrula stage. (16) Late gastrula stage. (17) Early neurula; head formation. (18) Late neurula; optic bud formation. (19) Six somite; brain and optic vesicle formation. (20) Nine somite; appearance of heart analogue. (21) Twelve somite; heart formation. (22) Sixteen somite; start of heartbeat. (23) Eighteen to nineteen somite; onset of blood formation. (24) Hatchlings (Bar = 0.5 mm).

embryonic observation under the microscope was done in a controlled environment (i.e. an enclosed room with an air conditioning system set at 28°C). Hence, it is believed that the environmental and water temperature during the study was substantially similar in all treatments as well as in all the experiments in the study (also confirmed with the mean water quality observed in the study).

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Table 1 Description of the embryogenesis of diploid and triploid developing eggs of *Anabas testudineus* under laboratory conditions (Temperature = 28.6 ± 1.1°C). Numbers are means hours:minutes

Stage no.	Stages	Diploid	Triploid	Description/Observation
1	Fertilized egg at the 1-cell stage	0:00	0:00	Upon fertilization, the eggs of *Anabas testudineus* expanded resulting in the separation of the inner yolk from its outer membrane. The adhesive nature of the eggs at this stage facilitated their attachment to the hatching substrate. After a few minutes, there was an accumulation of granulated cytoplasm at the anterior end of the yolk, thereby segregating the animal pole from the vegetal pole (forming the one eye stage or the blastodisc)
3	2-cell stage	0:30	0:30	The first mitotic division of the granulated cytoplasm (animal pole) to produce two equal cells
4	4-cell stage	0:45	1:30	The second meroblastic cleavage of the cells to form the '4-cell stage'
5	8-cell stage	1:00	2:10	The third cell division results in the '8-cell stage'
6	16-cell stage	1:30	2:20	The fourth parallel division of the previous eight cells results in the '16-cell stage'
7	32-cell stage	2:00	2:30	The cell divides for the fifth time to produce the '32-cell stage'
8	64-cell stage	2:30	2:45	Further cell cleavage producing the '64-cell stage', many of the divisions at this stage were basically latitudinal in nature
9	128-cell stage	3:00	3:40	The seventh division result in the production of 128 daughter cells of relatively small size. At this stage, cell tend to lie over one another, hence making counting extremely difficult
10	Morula stage	3:30	4:30	Further latitudinal cell divisions produced tiers of numerous tiny blastomeres
11	Early blastula stage	4:00	6:00	Further blastomere division leads to unclear borders, hence, resulting in cell spreading over the yolk
12	Middle blastula stage	5:00	7:00	At this stage, blastomeres spreading gradually reduce and flattened. Cell division also not synchronous and much more difficult to number
13	Late blastula stage	5:30	7:30	Epibolic cells increase and early germ ring appeared, blastocoels formation seen
14	Pre-early gastrula stage	6:00	8:00	Blastoderm becomes flattened down onto the yolk sphere
15	Early gastrula stage	6:30	9:00	Germ cell rings epiboly one-quarter of the yolk. Embryonic shield rudiment appears
16	Mid-gastrula stage	7:00	11:00	Germ rings epiboly half of the yolk sac. Embryonic shield and neural plate are formed
17	Late gastrulation stage	9:00	12:00	Germ rings epibolyed three-quarters of the yolk sac. Embryonic body rudiment is formed
18	Early nuerula stage (head formation)	10:00	13:00	Embryo yolk sphere is nearly covered by thin blastoderm. Neurocoele of cells is seen in front of the head, small vacuoles (Kupffer's vesicles) appear at the upper side of the caudal
19	Late neurula stage (optic bud formation)	11:00	14:00	Brain and nerve cord in the arrow-shaped embryonic body co-develop at this stage as a rod-like cells. Also, rudimentary eye vesicles appears on each side of the cephalic end
20	4-somite	12:00	16:00	A pair of auditory vesicles appeared. Also, three parts of the brain (fore, mid- and hindbrain) are discernible
21	6-somite (brain and optic vesicle formation)	13:00	17:00	Optic vesicles differentiate to form the optic cups, while the lenses begin to form. The three regions of the brain are now well defined and the neural fold (neurocoele) is seen in the median line along the body
22	9-somite (appearance of heart analogue)	14:00	18:00	Tubular heart appears underneath. The head from the posterior end of the mid-brain to the anterior end of the hindbrain, body cavity extends further toward the posterior end of the eye vesicles. Also, incomplete lenses are present in the eyes
23	12-somite (heart formation)	15:00	19:00	Straight-tubed heart reaches beneath the posterior end of the eye vesicle. The Kupffer's vesicles shrink, spherical optic lenses were also completed, while blood island becomes pronounced in the ventral region between the 6th and 11th somites

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Table 1. *Continued*

Stage no.	Stages	Diploid	Triploid	Description/Observation
24	16-somite (start of heart beat)	16:00	20:00	Heart start to beat at a low pulsation between 33–55 beats/min. At this point, Kupffer's vesicles have disappeared and the otoliths are yet to be present in the optic vesicles
25	18–19 somite (onset of blood formation)	17:00	21:00	The spherical blood cells are first pushed out of the blood island (7–15 somites toward the blood circulation) vitello-caudal vein. The blood is pumped (60–70 heart-beats/min) from the heart out into the anterior cardinal vein and the dorsal aorta roots
26	Hatching	18:00	23:00	Movement of the fully formed embryo against the chorion wall to force it to rupture

Fertilization rate in this study was determined when embryogenesis was at the 2-cell or 16-cell stage following the method proposed by Okomoda *et al.* (2018). Hence, upon discriminating the 'fertilized' and 'hydrated' eggs (by observing actively dividing animal pole), the fertilization rate was estimated as shown in the equation below;

$$\%\ \text{Fertilization} = \frac{\textit{Fertilized eggs in the Petri dish}}{\textit{Total number of eggs in the Petri dish}} \times 100$$

Hatchability percentage in this study was also determined using the equation below

$$\%\ \text{Hatchability} = \frac{\textit{no. of hatched larvae}}{\textit{total no. of spawned eggs}} \times 100$$

Descriptive statistics for breeding parameters were performed using Minitab 14® computer software followed by Student's *t*-test. Also, the status of triploidization was affirmed by observing the erythrocyte morphology of 2-month-old juveniles (mean weight = 5.6 ± 0.22 g) following the recommendation of numerous researchers (e.g. Karami *et al.*, 2010; Pradeep *et al.*, 2011; Normala *et al.*, 2016).

Results and Discussion

The fertilization percentages of the two groups of eggs were similar (~90%) in this study (Fig. 1). This result was not surprising as the treatments were from similar breeding stocks and the heat shock was applied only after fertilization was carried out. The early cleavage stages of the developing embryos for both diploids and triploids (Fig. 2 and Table 1) were mainly mitotic discoidal meroblastic divisions characterized by an incomplete cleavage which exclusively occurred in the animal pole (Buzollo *et al.*, 2011; Olaniyi and Omitogun, 2013). This is similar to previous observations in many different species, resulting in blastomeres of relatively equal sizes (Kimmel *et al.*, 1995; Aluko, 1995; Olufeagba *et al.* 1999, 2016 Arockiaraj *et al.* 2003; Ninhaus-Silveira *et al.*, 2006; da Rocha *et al.* 2009). Subsequently, embryonic cleavage occurred in latitudinal directions, resulting in tiny blastomeres often referred to as 'mulberry' or 'half-berry' or 'ball'-like shapes (Honji, *et al.*, 2012; Olaniyi & Omitogun, 2013). The relatively small sizes of these cells made them impossible to count. It is important to note that despite the similarities of the embryogenetic stages (i.e. beyond the 2-cell stage); the triploid eggs were quite slow to develop (15 min to 5 h late) when compared with the diploid eggs. It is believed that the three min heat shock applied approximately 4 min after fertilization for the triploidization process slowed down biological activities significantly. So, in addition to retention of the second polar body, embryonic development time was delayed to when the eggs hatched. This observation is at variance with the reports of Happe *et al.* (1988) for rainbow trout *Saho gairdneri*, as here triploid eggs hatched earlier than their control diploid group.

Aydın & Okumus (2017) had earlier described that abnormal cell cleavage in triploid black sea turbot (*Psetta maxima*) eggs was the main reason for low hatchability in their study. Other studies by Devauchelle *et al.* (1988), Kjørsvik *et al.* (1990, 2003) and Pickova *et al.* (1997) also demonstrated a positive correlation between high hatching rate and percentage of normal blastomere division. Contrary to these observations, abnormal cleavage was not observed on a large scale in this study and therefore it was not thought to be the main cause of the low hatchability observed (i.e. 50.04 versus 79.56% respectively for triploid and diploid eggs). However, mass mortality of eggs was recorded in the somite stages and was thought to be the most critical developmental stage in this study. Similar mass mortality has been reported previously by Okomoda *et al.* (2017) for progenies of ♀*P. hypophthalmus* × ♂*C. gariepinus* during the somite stages. Importantly, similar types of mortality were observed in the triploid eggs rather than in the diploid eggs. Therefore, while mortality in the study by Okomoda *et al.* (2017) could be explained by hybridization

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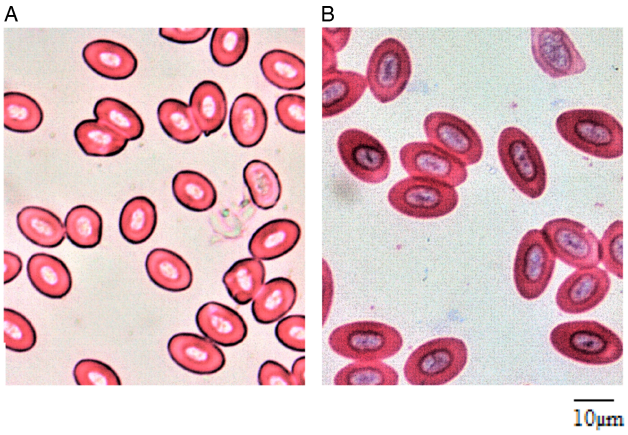

Figure 3 Erythrocyte morphology of (a) diploid; and (b) triploid *Anabas testudineus*. (Bar = 10 μm).

effects, the observations of the current study is more likely to be connected with the detrimental effect of the heat-shock process applied for triploidization.

Erythrocyte size variation between triploid and diploid eggs has been well established in many previous studies and has long been found pride of place as a simple index of discrimination. No matter the shape of the diploid erythrocytes (rounded or oval), triploids of the same age and size usually possess more than 1.5 times (and above) the erythrocyte major axis of their diploid counterparts (Lincoln & Scott 1983; Varadaraj & Pandian 1990; Koedprang & Na-Nakorn, 2000; Gao *et al.*, 2007; Dorafshan *et al.*, 2008; Karami *et al.*, 2010; Normala *et al.*, 2017). Although detailed biometrics of the erythrocytes of both diploid and triploids were not reported in this current study, visual observation of both groups affirmed the suitability of erythrocyte morphology as a simpler index of discrimination between the diploid and triploid *A. testudineus* in line with the findings of many previous studies (Fig. 3). According to Benfey (1999) and Normala *et al.* (2016), the increased erythrocyte size of the triploids is a significant biological response resulting from an increment in one chromosome set.

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Conflict of Interest

None

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