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ABNORMALITIES AND POSSIBLE MOSAICISM DURING EMBRYONIC CELL DIVISION AFTER COLD SHOCK IN ZYGOTES OF THE PACIFIC WHITE SHRIMP, *LITOPENAEUS VANNAMEI*, RELATED TO FAILURE OF INDUCTION OF TETRAPLOIDY AND TRIPLOIDY

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ABSTRACT

Abnormalities and possible mosaicism during embryonic cell division after cold shock of zygotes of *Litopenaeus vannamei* (Boone, 1931) were related to the failure of induction of polyploidy. Eggs were treated to 10 minutes cold shock at 8°C to arrest the second polar body (triploid-induced eggs) or first cleavage (tetraploid-induced eggs). In both cases, asynchrony and abnormalities among embryos were observed. In some triploid-induced eggs, three haploid pronuclei, instead of two, were seen at the center of the egg, and none in the periphery. These three nuclei may serve to evaluate methods of triploid induction in penaeids. In tetraploid-induced eggs, a tetrapolar or a bipolar spindle was inferred. The common abnormality in the anaphase with a tetrapolar spindle came with an off-center position of the nucleus, ending in a 2-cell stage with either a blastomere with one complete nucleus ($4n$) or two separate nuclei ($2n$ and $2n$), both with one anucleated ($0n$) blastomere. Other abnormalities produced 3- and 4-cell stages with one to three anucleated ($0n$) blastomeres, including one putative mosaic. In triploid-induced eggs, the main abnormalities came with syngami between two pronuclei only, ending in a putative 4-cell stage 1:1 triploid ($3n$): diploid ($2n$) mosaic egg or without syngamy, ending in a putative 4-cell stage 2:1:0 diploid 2($2n$): haploid (n): anucleated ($0n$) mosaic egg, and perhaps a putative 1:1 diploid ($2n$): haploid (n) mosaic egg. Less frequent 4-cell stage arrangements had 1:1 sextaploid ($6n$): anucleated ($0n$) and 1:3 one nucleated ($3n$) and three anucleated ($0n$) blastomeres. We suggest that inherent problems in *L. vannamei* embryos are related to lethal occurrences of putative nauplii mosaics. Alternative methods to induce polyploidy are needed for this species.

KEY WORDS: abnormalities, mosaics, syngamy, tetraploidy, triploidy

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INTRODUCTION

Induction of triploid shrimp confers sterility for genetic protection of prime stocks and skews sex ratios towards a high proportion of females, which are larger than males. This may lead to higher income in shrimp farming (see Sellars et al., 2010 for review). In *Fenneropenaeus chinensis* (Osbeck, 1765), *Marsupenaeus japonicus* (Bate, 1888), *Penaeus monodon* Fabricius, 1798, and other penaeid species, viable triploid larvae have been successfully induced and reached adulthood (see Sellars et al., 2010 for review, and Wood et al., 2011). Induction of viable triploid postlarvae in Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931) one of the most important species in shrimp aquaculture, has been unsuccessful. Cold shock induces a proportion of meiosis II triploid embryos (Dumas and Campos-Ramos, 1999; Sellars et al., 2012), and 6-dimethylaminopurine (6-DMAP) induces a proportion of meiosis I and II triploid embryos (Sellars et al., 2012). Other studies in triploid induction and even tretraploidy induction in *L. vannamei* claim to have optimized manipulation of chromosome sets and survival applying cold shock; however, they present no evidence of triploid and tetraploid chromosome counts or flow cytometry analysis (Garcina-Rivera et al., 2004; Alok et al., 2006; de Almeida et al., 2011). Heat shock of 38°C is lethal to *L. vannamei* embryos, with a threshold of 36°C (CIBNOR and ITSON, unpublished information). Heat shocks between 32 and 36°C need to be tested in *L. vannamei*, since this range has induced triploidy in *P. monodon* when spawned at 29°C (see Sellars et al., 2010 for review). Sellars et al. (2012) made a comprehensive study of induction of triploidy in *L. vannamei*, where still no viable postlarvae were produced, and repeatability was not consistent, even among similar time frames of cold shock and 6-dimethylaminopurine (6-DMAP) treatments. However, some of the treatments to induce triploid embryos reached triploid protozoeal stages, which now represents the best successful induction rates in *L. vannamei*. These authors provided a description of the ab-

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normalities observed in induced triploid *L. vannamei* with 6-DMAP and cold shock (Sellars et al., 2012).

Failure of induction of triploid *L. vannamei* has not allowed studies in sex determination, sex ratios, and especially, growth and survival rates that the shrimp industry demands. An alternative experimental approach could be cold or heat shock for the induction of tetraploids after arresting the first cleavage. When tetraploid shrimp are crossed with diploid shrimp, progeny are all triploid. Although tetraploid embryo induction is feasible in *F. chinensis* (Li et al., 2003) and *M. japonicus* (Sellars et al., 2006) using heat shock and *P. monodon* (Foote et al., 2012) using cold shock, no viable larvae have been produced. According to Morelli and Aquacop (2003) who tested *Fenneropenaeus indicus* (H. Milne Edwards, 1837) and Foote et al. (2010) who tested *M. japonicus*, heat shock produces cytological defects during embryogenesis that lead to death.

The technological and scientific horizon to solve this problem of polyploidy induction in *L. vannamei* appears to be distant; therefore, it is important to gather cytological information during embryogenesis that could lead to workable methods of polyploidy induction. Here, we show that abnormal mitosis and possible mosaicism during embryo cell division are related to the failure to induce tetraploidy and triploidy using cold shock, in contrast to normal development of diploid embryos.

Materials and Methods

Bioassays were made at a shrimp hatchery in Sonora, Mexico. Ripe female shrimp broodstock (approximately 40 g body weight), with a visible spermatophore adhering to the thelycum were individually placed in 50 l cylindrical containers filled with 30 l filtered seawater at 28.5-29°C and 34 g l^{-1} salinity. The female started to spawn (time zero), and approximately 2.5 minutes later, it was removed from the container. The volume of seawater was reduced to approximately 16 l by removing water through a 100 μm Nitex nylon mesh (Sefar, Heiden, Switzerland). The eggs were divided among six PVC cylinders: 3 for controls and 3 for treatments (approximately 1.5×10^4 eggs per PVC cylinder, 7.6 cm diam, 20 cm height, Nitex nylon mesh was secured at the bottom). The cylinders were placed inside a 150 l acrylic-sided aquarium with the same water conditions and with moderate aeration. Only spawns with a fertilization rate > 80% were analyzed, and hatching (%) was evaluated. Aliquots of approximately 30-50 eggs from one of the three control replicates and one from the three treatment replicates were taken randomly every minute during the first 60 minutes, starting at post-spawning time of 8 minutes, with the exception of the time when embryos were cold-shocked. Thereafter, samples were taken every 1 or 2 hours until hatching. Samples of eggs and embryos were immediately fixed with a fresh and cold 3:1 solution of ethanol to acetic acid (v/v), with two changes of the fixative (15 minutes each). The samples were stored at 4°C until processed. Before observing the samples, the fixative was changed to ethanol, spun for a few seconds, and the ethanol was decanted. Then, 25 μl 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA), was added to the samples and incubated for 10 minutes in the dark at 25°C, placed on slides, gently covered with a cover glass, and observed and photographed under a fluorescence microscope. Percentage of abnormalities was based on the 2- and 4-cell stages. Interpretation of embryonic development and abnormalities were based on Hertzler (2005a, b), Morelli and Aquacop (2003), Zhang et al. (2007), Biffis et al. (2009), Foote et al. (2010), Pongtippatee et al. (2012) and Sellars et al. (2012).

To analyze tetraploid-induced eggs, 18 spawns were used to cover the period before, during, and after the first mitotic metaphase. The metaphase occurs approximately 30 minutes post-spawning at 28°C (Hertzler, 2005b). Following initial culturing at 28.5-29°C, a cold shock at 8°C was applied at 24, 26, 28, 30, 32, or 34 minutes post-spawn for 10 minutes. Three spawns ($n = 3$ replicates) at each sample time were analyzed. Each spawning female was randomly picked and assigned a time for treatment. The cold shock procedure involved gently lifting and transferring three of the PVC cylinders (at random) into a 150 l acrylic aquarium at 8°C. The remaining three PVC cylinders (controls) were treated in the same way without transferring them. After treatment, treated eggs in the PVC cylinders were returned to the original tank that still contained the control cylinders at 28.5-29°C. To analyze cold shock for induction of triploidy, six spawns ($n = 6$ replicates) were used to arrest the second polar body (meiosis II triploid embryos). Cold shock at 8°C was applied at 10 minutes post-spawning for a duration of 10 minutes, based on Dumas and Campos-Ramos (1999) and unpublished information. The procedures are described above.

Results

Hatching and Abnormalities in Tetraploid-Induced and Triploid-Induced Eggs

In tetraploid-induced embryos, a delay of one cell-stage, and a mean of 89% with half of the body anucleated occurred in treatments at 28, 30 and 32 minutes post-spawning (around first mitotic metaphase-anaphase), and did not hatch. The remaining 11% were unfertilized eggs. Treatments before (24 and 26 min) and after (34 min) had no abnormalities, and development was similar when compared to controls, but with lower hatching (mean of treatments 37%; mean of controls 56%) (Table 1). In triploid-induced embryos, a delay of one cell-stage, a mean of 30% with half of the body anucleated, plus 10% unfertilized eggs, and lower hatching than controls (28 and 52%, respectively) occurred (Table 1). The remaining 60% were apparently normal embryos. However, the hatched naupllii died before metamorphosis to the protozoea stage.

Pronuclei in Oöplasm of Tetraploid-Induced and Triploid-Induced Eggs

Eggs at anaphase of the second meiotic division (10-13 minutes post-spawning) are shown in Fig. 1 (Control 1.1A, tetraploid-induced 1.2A and triploid-induced 1.3A). Subsequently in control and tetraploid-induced eggs, two haploid pronuclei were defined in the center of the egg, whereas the second polar body remained in the periphery (Fig. 1.1B, 1.2B), followed by prophase (Fig. 1.1C, 1.2C), and metaphase (Fig. 1.1D, 1.2D). Some triploid-induced eggs showed three haploid nuclei, instead of two, in different arrangements in the oöplasm (Fig. 1.3B-F) and none in the periphery. The mean of eggs with three pronuclei ($3n$) was 34%. However, if only a triangle arrangement is considered (40% of all eggs with three nuclei), the mean of eggs with three pronuclei was 12%. The mean of eggs prior to first cleavage, either in prophase (Fig. 1.3G) or metaphase (Fig. 1.3H), without a distinguishable second polar body in the periphery was 42% (Table 1).

Tetraploid-Induced Eggs

Eggs during metaphase and early anaphase (28, 30 and 32 minutes post-spawning) had nuclear material stretched along the two blastomeres in all cases (Fig. 2A-C); thus, cytokinesis of first cleavage was not inhibited. From an inferred tetrapolar spindle, 70% of eggs at a 2-cell stage had an off-center nucleus: 1:1 nucleated ($4n$): anucleated ($0n$), and developed into two 4-cell stage abnormalities: two nucleated ($4n$) and two anucleated (0) blastomeres (Fig. 2D-G), with a mean of 66% and either a tetra-nucleated

Table 1. Percent (mean ± SD) of fertilization, hatching, and abnormalities in control and treated (cold-shock) eggs of *Litopenaeus vannamei*. Tetraploid-induced eggs ($n = 3$ spawns/minutes, post-spawning treatment), triploid-induced eggs ($n = 6$ spawns) and mean ± SD percentages of three pronuclei in the ooplasm, three pronuclei with a triangle arrangement in the ooplasm, and with no polar body observed in the periphery.

Minutes post-spawning	Fertilization (%)	Control eggs	Tetraploid-induced eggs		Triploid-induced eggs		Eggs with three pronuclei in the ooplasm		
		Hatch (%)	Hatch (%)	Abnormalities in non-hatched nauplii (%)	Hatch (%)	Abnormalities in non-hatched nauplii (%)	Three pronuclei (%)	Triangle arrangement (%)	No polar body observed (%)
10	90 ± 2.08	52 ± 1.78			28 ± 2.33	30 ± 3.48			
24	90 ± 2.08	56 ± 2.00	38 ± 2.51	0					
26	90 ± 1.00	57 ± 1.52	36 ± 1.52	0					
28	90 ± 0.57	54 ± 3.60	0	86 ± 2.00					
30	90 ± 1.00	55 ± 2.08	0	90 ± 2.00					
32	90 ± 1.00	57 ± 1.52	0	92 ± 2.00					
34	90 ± 1.00	58 ± 1.52	37 ± 1.15	0					
Mean	90 ± 1.02	56 ± 2.07	37 ± 1.00	89 ± 3.05					
							34 ± 2.82	12 ± 1.50	42 ± 4.96

($n + n + n + n$) blastomere (Fig. 2H) or one nucleated ($4n$) blastomere (Fig. 2I), both with three anucleated ($0n$) blastomeres with a mean occurrence of 6%. The remaining 30% of eggs at the 2-cell stage had two separated nuclei off-center: 1:1, 2($2n$):($0n$) (Fig. 2J-L) and apparently few eggs with this condition formed a tetrapolar spindle, which developed into a 4-cell stage 1:1:0:0 tetraploid ($2n + 2n$): diploid ($2n$): anucleated 2($0n$) mosaic (Fig. 2M) with a mean occurrence of 4%. We inferred that most of these remaining eggs formed a bipolar spindle and developed into a 4-cell stage with three nucleated ($2n$) blastomeres and one anucleated (0) blastomere (Fig. 2N) with a mean occurrence of 8%, a 3-cell stage with two nucleated ($4n$) blastomeres and one anucleated ($0n$) blastomere (Fig. 2O) with a mean occurrence of 10%, and a 3-cell stage with one nucleated ($4n$) blastomere and two anucleated ($0n$) blastomeres (Fig. 2P) with a mean occurrence of 6%.

Triploid-Induced Eggs

In triploid-induced eggs, the most frequent abnormality at the 2-cell stage, with a mean occurrence of 60%, was when syngamy apparently took place only between two pronuclei and continued to mitosis (Fig. 3A-C), segregating one nucleus ($2n$) in each blastomere and leaving the third pronucleus (n) aside in one of the blastomeres by a random process. Fusion took place between the diploid ($2n$) and haploid (n) nuclei, resulting in a theoretical 4-cell stage 1:1 triploid ($3n$): diploid ($2n$) mosaic egg (unknown percentage). In another case, syngamy apparently did not occur, leaving a 2-cell stage with one ($n + n$) nucleated and one (n) nucleated blastomere (Fig. 3D), with a mean occurrence of 30%, which after the second cell division (4-cell stage), fusion of $n + n$, and mitosis occurred and appears to have become three nucleated and one anucleated blastomeres: two $2n$, one n and $0n$, a mosaic (Fig. 3E), with a mean occurrence of 62%, and perhaps, a variant of this arrangement developed into a theoretical 4-cell stage 1:1 triploid ($2n$): haploid (n) mosaic egg (unknown percentage). Another abnormality was an off-center nucleus at the 2-cell stage with a 1:1, either triploid ($3n$) or sextaploid ($6n$): anucleated ($0n$) (Fig. 3F), with a mean occurrence of 10%. The following 4-cell stages showed two nucleated ($6n$) and two anucleated ($0n$) blastomeres (Fig. 3G), with a mean occurrence of 12%, and one nucleated ($3n$) and three anucleated ($0n$) blastomeres (Fig. 3H), with a mean occurrence of 26%.

Gastrulation in Tetraploid-Induced and Triploid-Induced Eggs

In tetraploid and triploid-induced eggs, most of the abnormal eggs stopped cell division and died during abnormal gastrulation with half of the body anucleated (Fig. 4A-D and 4E-H, respectively).

Discussion

In triploid-induced eggs, three pronuclei were identified around the center or off-center in the oöplasm, which corresponds to the female and male pronuclei and the arrested second polar body. Eggs with three pronuclei centered and arranged in a triangle may have successful triploidization (Fig. 5A), whereas those with separated pronuclei do not. Further research is needed. Pongtippatee et al. (2012) observed eggs with three pronuclei in triploid *P. monodon* and showed that the percentage of eggs without a second polar body extrusion was higher than the percentage of eggs with three nuclei. Our results showed the same pattern. In theory, the percentages should be similar; thus, an over-estimate of triploidy should be avoided.

Most of the tetraploid-induced abnormalities in penaeids are well documented and follow the descriptions of Morelli and Aquacop (2003) and Foote et al. (2010) and in fish by Zhang et al. (2007), where poly-polar spindles form without a cell division checkpoint, and anucleated blastomeres continue dividing. According to Foote et al. (2010), the first cleavage was suppressed and a tetrapolar cleavage occurred. Morelli and Aquacop (2003) also described these two events, but in addition, observed eggs in which the first cleavage was suppressed but continued as a 2-cell stage containing a large single nucleus in one blastomere – an event that matches our observations. However, we suggest that the first cleavage was not inhibited because of the stretching or pulling off all the nuclear material towards

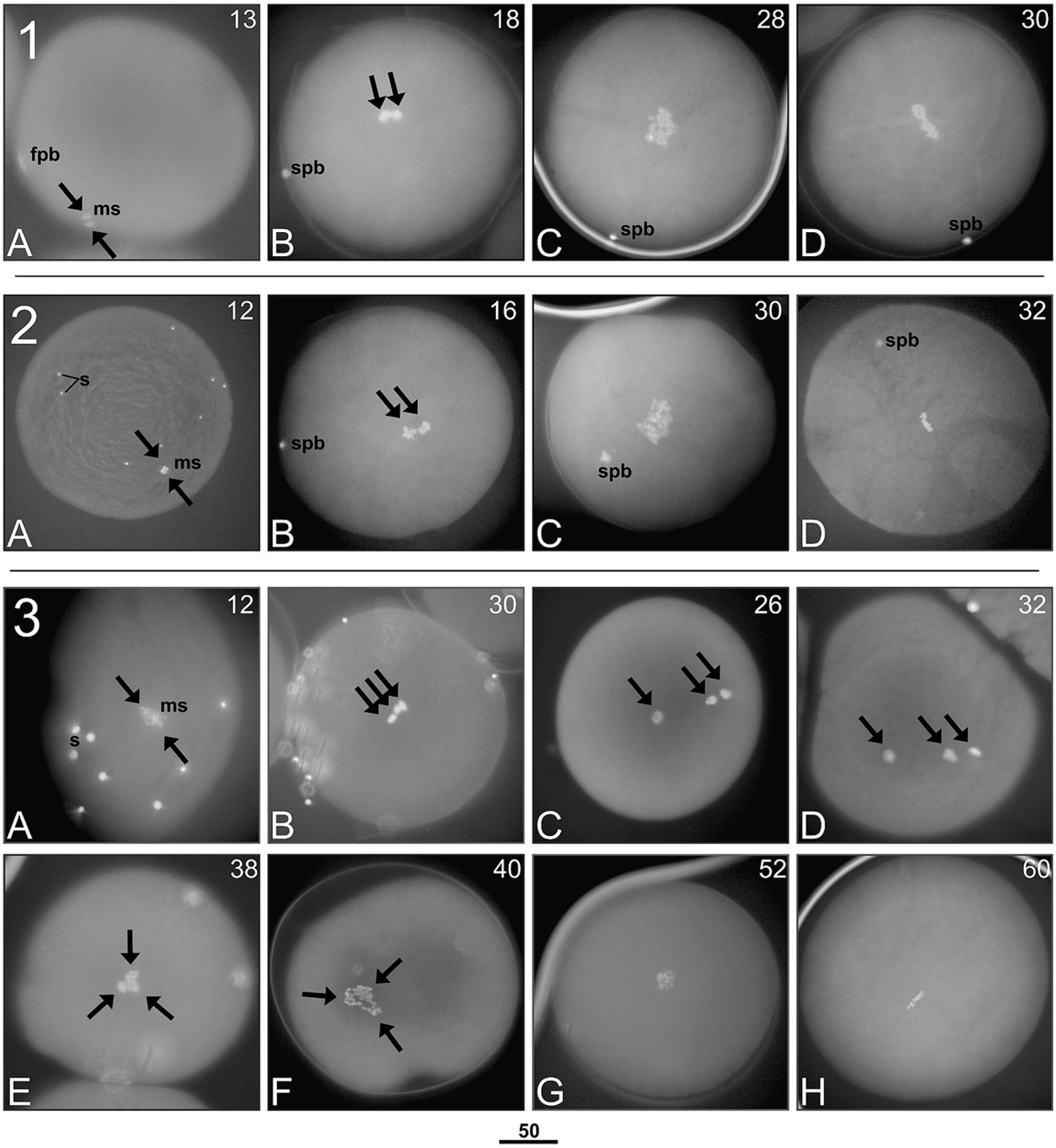

Fig. 1. Second meiotic division, prophase and metaphase at 28°C in *Litopenaeus vannamei* oöcytes stained with 4′,6-diamidino-2-phenylindole (DAPI). Time of post-spawning (in minutes) is indicated at the upper right of each microphotograph. 1A-D, control eggs; 2A-D, tetraploid-induced eggs; 3A-H, triploid-induced eggs. During the second meiotic division, two female nuclei were forming a meiotic spindle (ms) in the periphery and were oriented in parallel to the surface of the egg (1A-3A). Control eggs (1A) had an extruded first polar body (fpb), and chromosome segregation (arrows in panels 1A-3A). Sperm (s) trapped in the hatching envelope of the oöcyte are shown in 2A and 3A. After pronuclear migration in control and tetraploid-induced eggs, two haploid pronuclei were present in the center of the egg (1B and 2B; arrows), and the second polar body (spb) remained in the periphery, including the prophase (1C and 2C), and metaphase (1D and 2D). In triploid-induced eggs, three haploid nuclei (arrows) were present in the center of the egg (3B and 3E), nearly centered (3C-D), or off-center (3F), and none of them in the periphery, including prophase (3G) and metaphase (3H). In some cases, the three pronuclei were together in the center (3B), in others, two were together and the third separated (3C), in others, the three pronuclei were separated from each other (3D), and in others, the three pronuclei were together, forming a triangle (3E-F). Bar represents 50 μm.

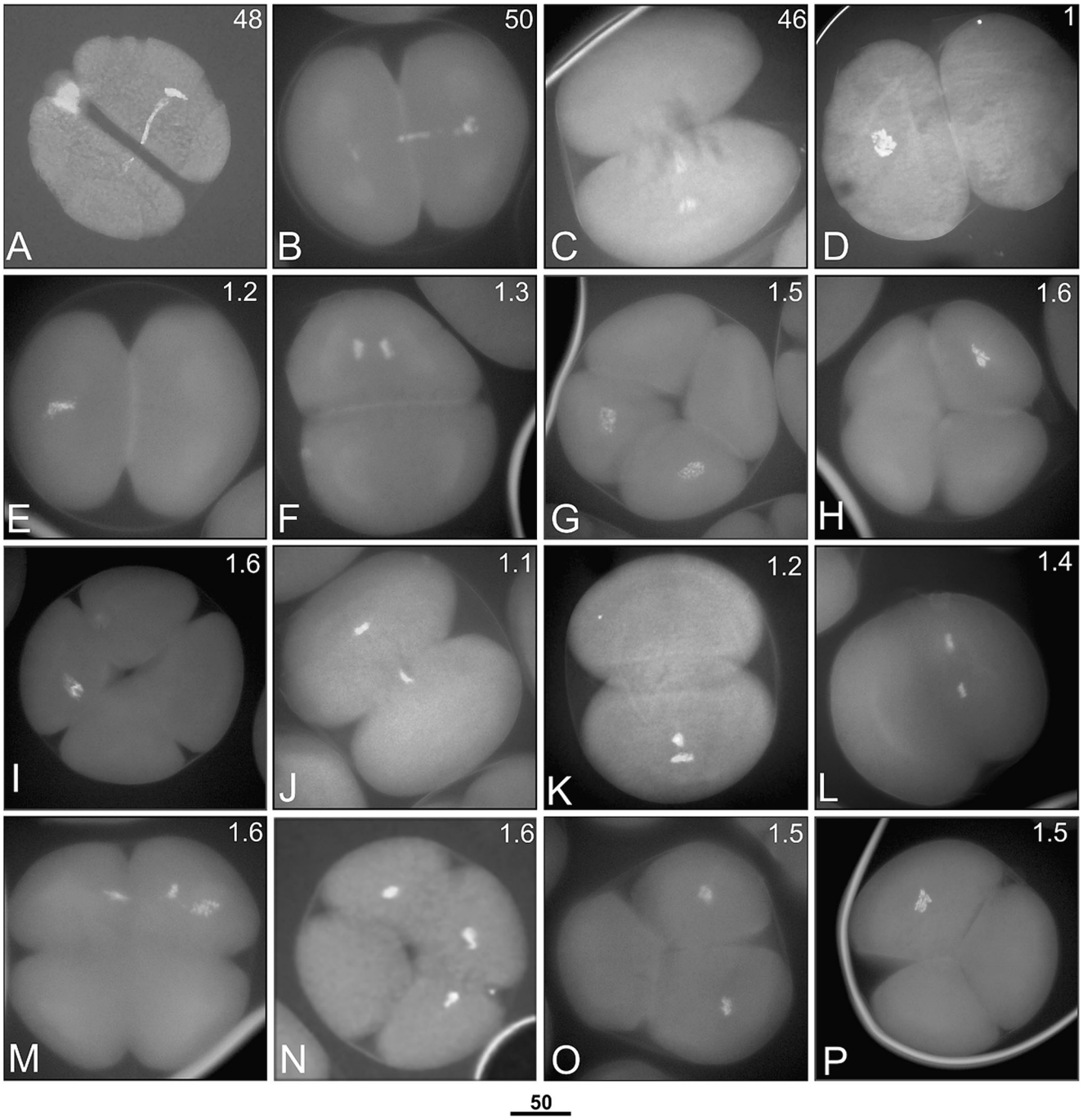

Fig. 2. Abnormalities in 2- and 4-cell stages in *Litopenaeus vannamei* tetraploid-induced eggs stained with DAPI and treated with cold shock either at 28, 30, or 32 minutes post-spawning. Time (up to 59 minutes, then in hours), post-spawning is indicated at the upper right of each microphotograph. A-C, Nuclear material was stretched along the two blastomeres during anaphase, which produced an off-center position of the nucleus (or nuclei); D, results from an inferred tetrapolar spindle produced a 1:1, nucleated ($4n$): anucleated ($0n$) egg that reached first cleavage; E, second mitotic cycle showing metaphase; F, anaphase; G, ending in a 4-cell stage, with half of blastomeres anucleated. H, a 4-cell stage variant showing either one apparently tetra-nucleated ($n + n + n + n$) blastomere and three anucleated ($0n$) blastomeres or I, a 4-cell stage showing one nucleated ($4n$) blastomere and three anucleated ($0n$) blastomeres. J; a 1:1, 2($2n$): anucleated ($0n$) arrangement at the 2-cell stage: instead of one nucleus off-center, there was a nucleus ($2n$) "trapped" in the middle of the two blastomeres and one nucleus ($2n$) at the center; K, two separated nuclei ($2n$) were in asynchrony, one in metaphase with the wrong orientation and the other in early prophase; L, both nuclei were separated with the wrong orientation in metaphase; M, a putative 4-cell stage 1:1:0:0 tetraploid ($2n - 2n$): diploid ($2n$): 2($0n$) anucleated, a mosaic. N, results from an inferred bipolar spindle produced a 4-cell stage with three nucleated blastomeres ($2n$) and one anucleated ($0n$) blastomere; O, a 3-cell stage with two nucleated ($4n$) blastomeres and one anucleated ($0n$) blastomere; P, a 3-cell stage with one nucleated ($4n$) blastomere and two anucleated ($0n$) blastomeres. Bar represents 50 μm.

one blastomere, which are the causes of all abnormalities described and created an entire off-center nucleus or two separated nuclei off-center (one of them with a deleterious condition, i.e., aneuploidy).

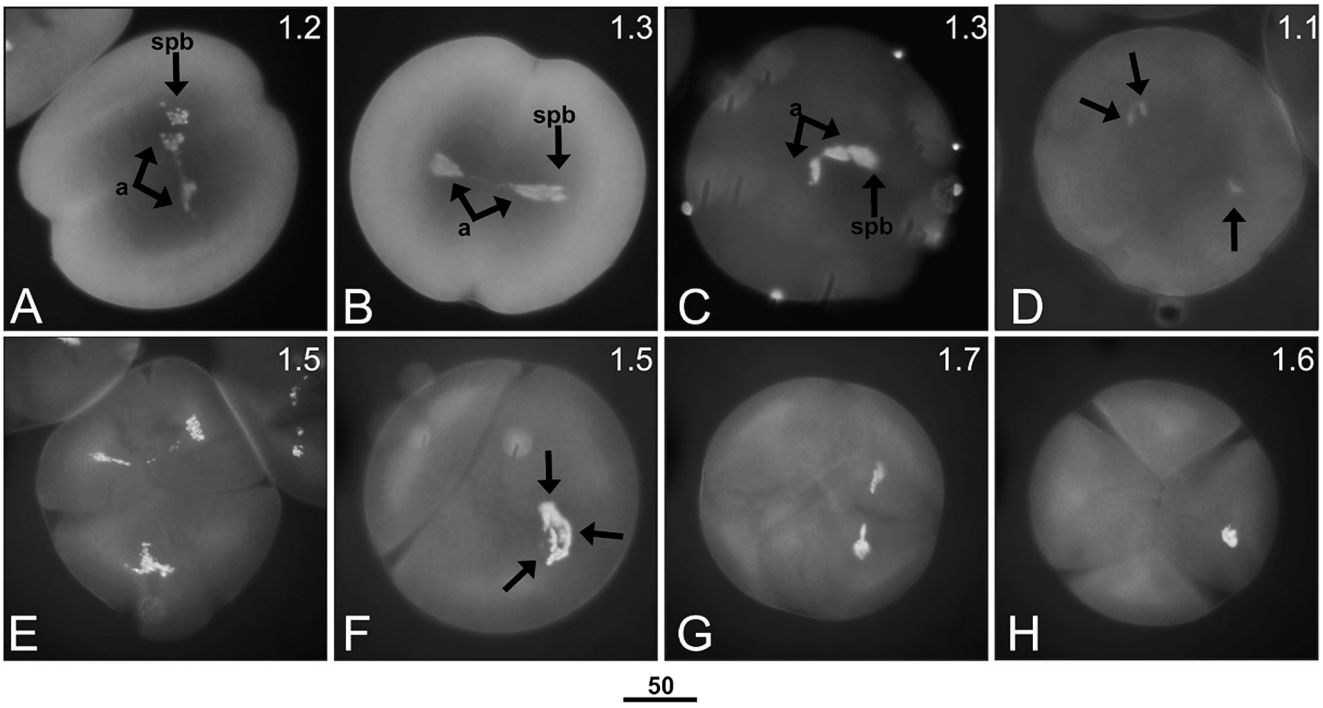

Fig. 3. Abnormalities in 2- and 4-cell stages in *Litopenaeus vannamei* triploid-induced eggs stained with DAPI and treated with cold shock at 10 minutes post-spawning. Time (hours) post-spawning is indicated at the upper right of each microphotograph. A-C, syngamy took place between two pronuclei only and anaphase (a) segregated one nucleus ($2n$) in each blastomere (arrows), leaving the third pronucleus (n), perhaps the second polar body (spb, arrow) aside in one of the blastomeres by random process. D, syngamy did not occur, leaving a 2-cell stage with one nucleated ($n+n$) and one nucleated (n) blastomeres; E, after the second cell cycle, it became a 4-cell stage with three nucleated and one-anucleated blastomeres: two were $2n$, one was n and another was $0n$, theoretically, a 2:1:0 diploid $2(2n)$: haploid (n): anucleated ($0n$) mosaic. F, a 2-cell stage with a 1:1, triploid ($6n$): anucleated ($0n$); G, the 4-cell stage had two nucleated ($6n$) and two anucleated ($0n$) blastomeres; H, a 4-cell stage with one nucleated ($3n$) and three anucleated ($0n$) blastomeres. Bar represents 50 μm.

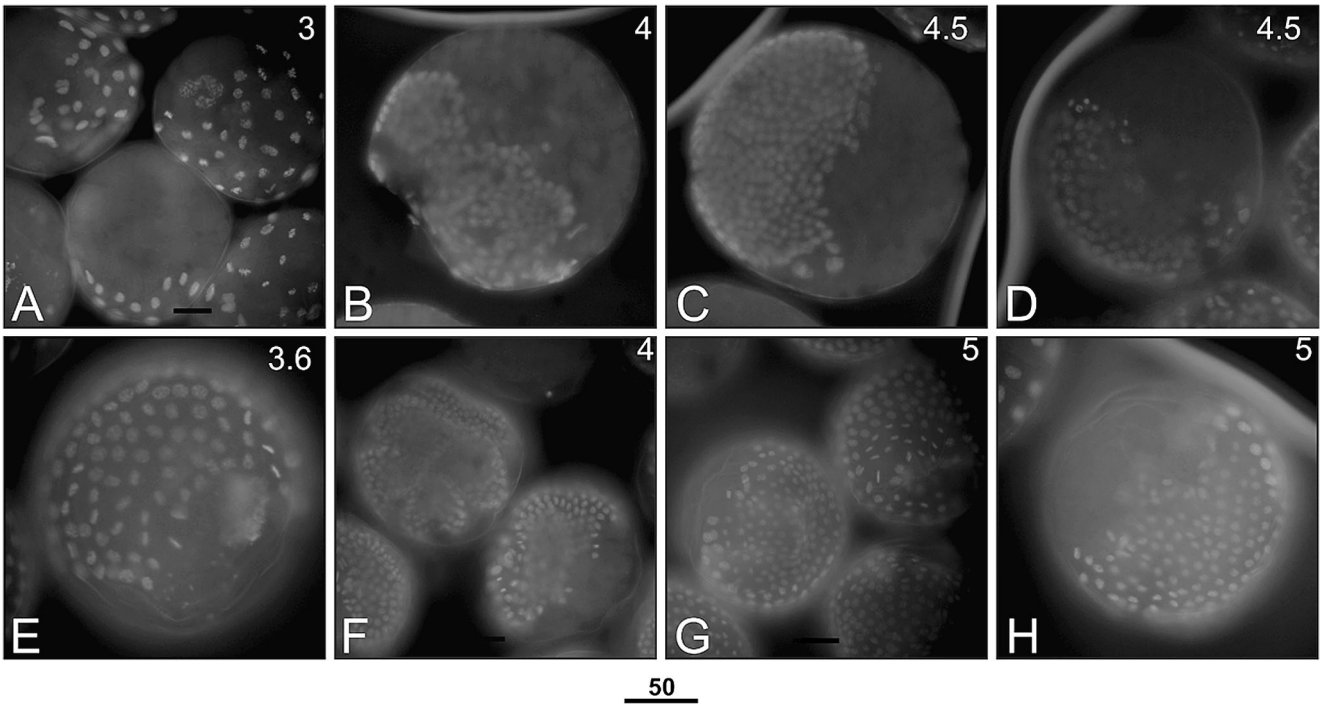

Fig. 4. Abnormal development of polyploidy-induced embryos around blastula-gastrula in *Litopenaeus vannamei* stained with DAPI. Embryos were nearly half anucleated, and did not hatch. Time (hours) after post-spawning is indicated at the upper right of each microphotograph. A-D, Tetraploid-induced eggs; E-H, triploid-induced eggs. Bar represents 50 μm.

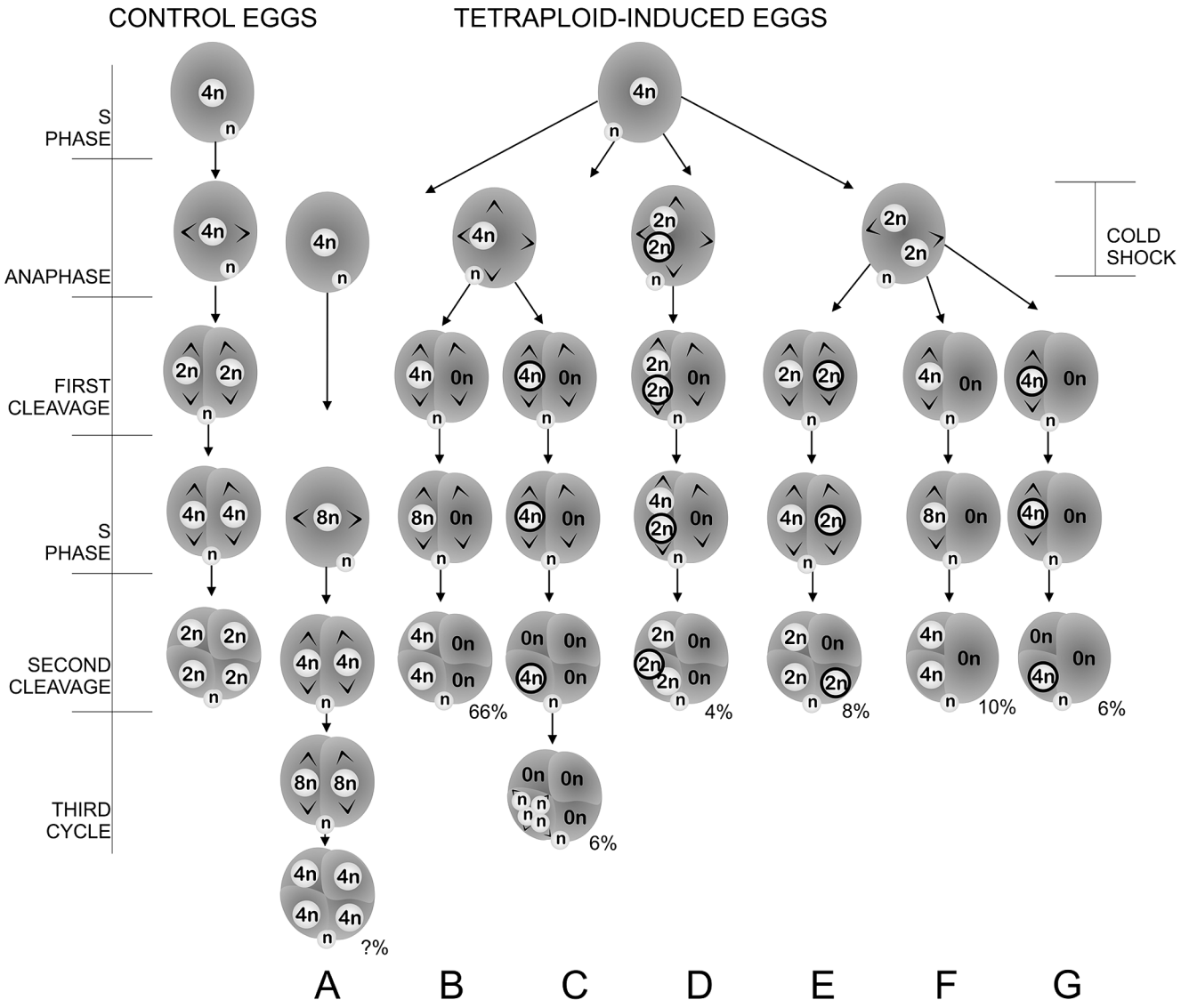

Fig. 5. Interpretations of tetraploid-induced development to the 4-cell stage. A, A true tetraploid suppressing the first cleavage (not induced); B-D, derived from tetrapolar spindles; E-G, derived from bipolar spindles. The percentage of occurrence is shown under each 3 or 4-cell stage. A, An ideal tetraploid egg; B, two nucleated 2(4*n*) and two anucleated 2(0*n*) blastomeres; C, one nucleated (4*n*) and three anucleated 3(0*n*) blastomeres; D, putative one tetraploid (2*n* + 2*n*), one diploid (2*n*) and two anucleated 2(0*n*) blastomeres, a mosaic; E, three nucleated 3(2*n*) and one anucleated (0*n*) blastomeres; F, two nucleated 2(4*n*) and one anucleated (0*n*) blastomeres; G, one nucleated (4*n*) and two anucleated 2(0*n*) blastomeres. Black circle in nucleus denotes inactivation-isolation. Haploid (*n*), diploid (2*n*), tetraploid (4*n*) and octaploid (8*n*).

In our study, a normal or a tetrapolar spindle was inferred before first cytokinesis, and depending on the type of spindle, a different 3- or 4-cell stage developed (Fig. 5). However, a true tetraploid egg (4*n*) was not induced (Fig. 5A).

From an inferred tetrapolar spindle, an off-center nucleus was confined to one of the two blastomeres, which developed into the most frequently observed 4-cell stage, where half of the blastomeres were nucleated (4*n*) only (Fig. 5B). Finding only one nucleated (4*n*) blastomere in a 4-cell stage may have developed because the nucleus, during the second cycle, had a deleterious condition (inactivated), and was pulled towards one blastomere ("isolation"), leaving the opposite blastomere anucleated (0*n*) (Fig. 5C). During the third cycle, the nucleus might have split into a four haploid (*n*) nuclei in the same blastomere, perhaps by a late formation of a tetraspindle, leaving a tetra-nucleated blastomere (last egg in Fig. 5C).

Few eggs at the 2-cell stage, with two separated nuclei (2*n*) in one blastomere (off-center), appeared to form a tetrapolar spindle, in which one of the nuclei proceeded with mitosis, leaving one daughter nucleus (2*n*) in the two blastomeres (although one of them is off-center), plus one nucleus (2*n*) in one of them inactivated and isolated, ending in a 4-cell stage: 1:1:0:0 tetraploid (2*n* + 2*n*): diploid (2*n*): anucleated 2(0*n*) (Fig. 5D). This type of abnormal mosaic would not be distinguishable from the tetraploid (4*n*)-anucleated (0*n*) egg during abnormal gastrulation and appears to be unique. According to Foote et al. (2012), some mosaics appeared in *P. monodon* during induction of

tetraploidy. Further research, using both flow cytometry and egg-embryo analyses is necessary.

We inferred that most of the eggs at the 2-cell stage with two separated nuclei ($2n$) in one or two blastomeres formed a bipolar spindle. The 4-cell stage, with three nucleated blastomeres ($2n$) and one anucleated ($0n$) blastomere arrangement, apparently developed after an abnormal first anaphase, where one nucleus ($2n$) was present in each blastomere (2-cell stage), but only one of them was deleterious (inactivated). This inactivated nucleus became isolated during the second cell cycle in one blastomere. In the opposite blastomere, the other nucleus ($2n$) continued with normal mitosis, and produced two diploid daughter nuclei (Fig. 5E). This is the case where the cold shock maintained diploid blastomeres in three-fourths of the eggs, and could not be distinguished from an abnormal tetraploid ($4n$)-anucleated ($0n$) during gastrulation, if the detrimental nucleus remained isolated in further divisions. This is supported by the absence of embryos in gastrulation with a one-fourth anucleated body. The variant 3-cell stages with bipolar spindles may have developed because the anucleated ($0n$) blastomere did not divide during the second cycle (absence of a previous tetrapolar spindle formation) and developed with two nucleated ($4n$) and one anucleated ($0n$) blastomere (Fig. 5F) or a 3-cell stage with one nucleated blastomere ($4n$), inactivated and isolated, and two anucleated ($0n$) blastomeres (Fig. 5G). Eggs treated before (24 and 26 minutes post-spawning) and after (34 minutes post-spawning) of the first metaphase-anaphase showed no abnormalities, but lower survival rates still require an explanation.

Abnormalities in tetraploid induction were not previously investigated in *L. vannamei*. It is clear that cold shock leads to abnormal blastomeres in all eggs, and it is not suitable for viable tetraploid larvae in this species. Therefore, alternative methods must be developed.

In triploid-induced eggs undergoing syngamy, presumably between the female and male pronuclei only, the arrested second polar body was probably left aside in one of the blastomeres by random action, suggesting that a normal first anaphase occurred, but leaving one diploid blastomere ($2n$) and one blastomere ($2n + n$). During the second cycle, fusion of nuclei ($2n - n$) in one of the blastomeres may take place, ending in a 4-cell stage mosaic, with two triploid ($3n$) blastomeres and two diploid ($2n$) blastomeres (Fig. 6B). In our study, this putative mosaic would be cytologically indistinguishable from a normal diploid ($2n$) egg or true triploid ($3n$) egg (if induced), when the three nuclei accomplished triangular syngamy in the center of the egg and proceeding with the first mitotic division (Fig. 6A). This putative mosaic was the most frequent (2-cell stage) in triploid-induced eggs, and its presence implies that, if only (or mostly) this mosaic would be formed in a given treatment, flow cytometry analysis would result in approximately 50% success of triploidization, when in fact, it would be almost a 100% mosaic. This example showed the importance of analyzing a given treatment by both egg and embryo cytology and flow cytometry. If fusion of nuclei ($2n + n$) does not take place and the haploid nucleus (n) remains isolated in one blastomere, it would end in a normal diploid ($2n$) embryo with a single triploid ($2n + n$) somatic cell. Presumably, this would be a non-lethal condition that did not occur in this study because all nauplii died.

The most abnormal and intriguing egg was a 4-cell stage with only three nucleated blastomeres, theoretically a 2:1:0, diploid 2($2n$): haploid (n): anucleated ($0n$) mosaic (Fig. 6C). This mosaic may have occurred without syngamy; subsequent cytokinesis of the first cleavage randomly separated (50/50 chance) two pronuclei occurring in one blastomere and one pronucleus in the other blastomere. The blastomere containing two pronuclei (presumably the female and male pronuclei) became diploid ($2n$) after fusing. In the second cycle, it divided to form the 4-cell stage, with two diploid ($2n$) blastomeres. The haploid (n) pronucleus (presumably the second polar body) contained in the other blastomere apparently remained inactivated and isolated; thus, one blastomere ended as an anucleated ($0n$) cell at the 4-cell stage. If the female or male haploid pronucleus (n) was left in one blastomere and further divided in the second cycle, a theoretical 1:1, diploid ($2n$): haploid (n) mosaic egg would arise (Fig. 6D) that would not be distinguishable in our study. Further confirmation is required.

In fish (Zhang et al., 2007) and shrimp (Foote et al., 2010), centrioles are not present in the meiotic spindle; thus, formation of poly-polar spindles after thermal shock, by the time of the second meiotic division, does not occur. This supports the conclusion that an anucleated ($0n$) blastomere in the putative mosaic "6C" did not have this origin. However, this does not apply to the abnormal half-anucleated ($0n$) eggs (2- and 4-cell stage): two nucleated ($6n$) and two anucleated ($0n$) blastomeres (Fig. 6E), and one inactivated and isolated nucleated blastomere ($3n$) and three anucleated ($0n$) blastomeres (Fig. 6F), which were similar in origin to the more typical eggs in tetraploid induction. One explanation is that, when these eggs were returned to the normal incubation temperature (28°C), their "recovery" was quite slow, which allowed further formation of tetrapolar spindles. This requires further confirmation.

In general, our study supports observations of a highly disorganized cleavage pattern and anucleated cells in embryos of triploid-induced *L. vannamei* (Sellars et al., 2012).

Observed abnormalities of triploid-induced embryos and death during gastrulation originated from "6E" and the mosaic "6C", if it developed as a 1:1, diploid ($2n$): anucleated ($0n$) (if the inactivated nucleus remains isolated in further divisions, since no embryos in gastrulation were observed with a one-fourth anucleated body). These recognizable abnormalities provide deeper knowledge concerning the failure to produce triploid embryos in *L. vannamei*, as previously studied by Sellars et al. (2012) in the same species. The origin and low proportion of abnormalities, when compared to the high proportion of "normal" or putative triploid-hatched nauplii that died before the protozoea stage, requires another explanation. We suggest that both the putative nauplii 1:1 triploid ($3n$): diploid ($2n$) mosaic (Fig. 6B) and, although less likely, the putative $2(2n) - 2(n)$ mosaic (Fig. 6D), are produced after cold shock and are lethal genotypes in *L. vannamei* larvae.

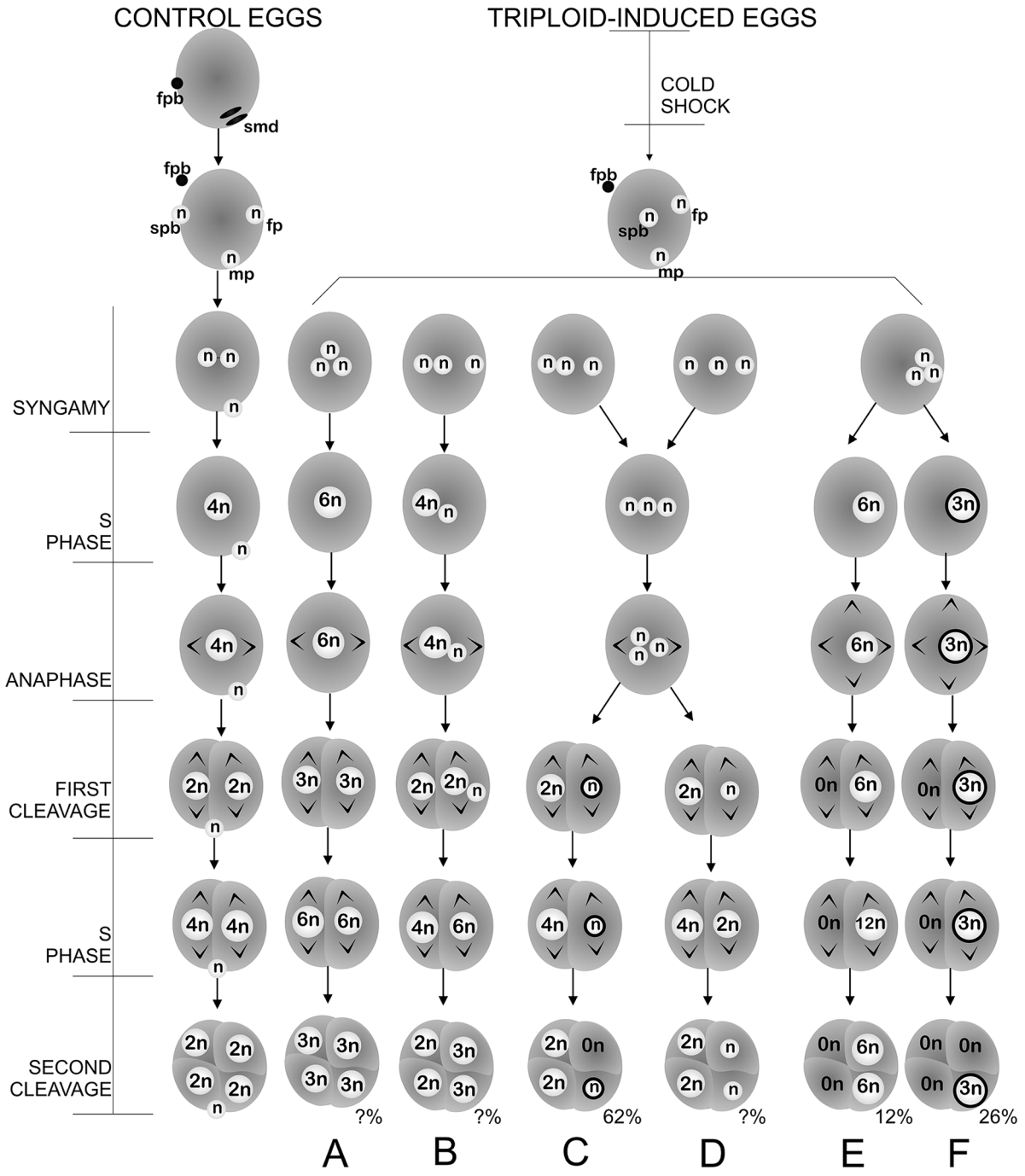

Fig. 6. Interpretations of triploid-induced development at the 4-cell stage. The percentage of occurrence is shown under each 4-cell stage. A, Putative triploid (3*n*); B, putative two nucleated 2(3*n*) and two nucleated 2(2*n*) blastomeres, a mosaic; C, two nucleated 2(2*n*), one nucleated (*n*) and one anucleated (0*n*) blastomeres, a mosaic; D, putative two nucleated 2(2*n*) and two nucleated 2(*n*) blastomeres, a mosaic; E, F, putative tetrapolar spindle forming a 4-cell stage, E, two nucleated 2(6*n*) and two anucleated 2(0*n*) blastomeres, F, one nucleated (3*n*) and three anucleated 3(0*n*) blastomeres. Black circle in nucleus denotes inactivation-isolation. Second meiotic division (smd); female pronucleus (fp); male pronucleus (mp); first polar body (fpb); second polar body (spb). Haploid (*n*); diploid (2*n*); triploid (3*n*); sexaploid (6*n*); dodecaploid (12*n*).

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