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A. N. Khanaychenko, Ph. D, S. Sc.

A. O. Kovalevski's Institute of Biology of the southern Seas of National Academy of Sciences of Ukraine, Sevastopol, Ukraine

SPECIES-SPECIFIC DIFFERENCES IN DIATOM-INDUCED ANOMALIES IN CALANOID COPEPOD EMBRYOGENESIS: ARE THEY LINKED TO THE STOCK-PILED ANTIOXIDANTS?

Egg production and viability of co-occurring in nature copepods, *Calanus helgolandicus* and *Calanoides carinatus,* fed diatom, *Thalassiosira rotula,* and then switched to dinoflagellate *Prorocentrum minimum*, were compared in experimental conditions. Egg production of both species on diatom diet was similar (up to 17-22 eggs.female-1.d-1) but no viable nauplii were observed. Development was arrested at different stages depending on degree of anomalies. Abnormal embryos displayed various degrees of deteriorations in pigment distribution and organization of extracellular matrix. Reproductive responses of *C.carinatus* both to negative (diatom) and positive (dinoflagellate) diets were postponed in comparison with quick responses of *C.helgolandicus*. After 3 days of dinoflagellate diet *C.carinatus* still produced only abnormal embryos with strong residual effect of diatom diet, while *C.helgolandicus* produced 50 % of viable nauplii. Based on own and literature data, our hypothesis links degree of diatom-induced copepod embryonic anomalies to the disturbances in antioxidant properties of the membranes and the degree of lipid peroxidation in embryo membranes and cytoplasm attributed to imbalanced content and ratio of HUFAs and carotenoids from freshly assimilated diet. Species-specific differences in copepod reproductive responses to diatom feeding are supposedly related to different stock-piled material and pathways of these essential components to the late oocytes.

Key words: calanoid copepods, diatom, embryo, anomaly, extracellular matrix, pigment

Since the start of copepod investigations till now, diatoms, usually dominating in natural phytoplankton of temperate waters during winter, early spring and autumn, are considered as one of the basic food components for the herbivorous calanoids [11, 42] and thus, the basis for their recruitment [3, 18]. Still, the field and experimental data of the last decade came in contradiction to this classic view. It appears that food web "diatoms-copepods" in many cases leads to reproductive failure of copepods. Several species of diatoms actively consumed by ovigerous females of copepods were proved to induce high egg production coinciding with low egg-hatching success resulted from abnormal embryo development [17, 31, 48, 52]. Low value of diatoms for copepod recruitment was observed during rearing of calanoid copepods through numerous generations [23, 25, 53]. Diatom-induced inhibition of reproduction in 16 copepod species was registered in 12 different environments by 15 laboratories [4]. Reproductive failures of *Acartia clausi* [17, 38, 39], *Calanus helgolandicus* [17, 37, 39], *C. finmarchicus* [52], *C. pacificus* [56], *Centropages typicus* [48], *Temora stylifera* [38] were observed in natural environment during diatom blooms. A similar effect was registered in laboratory conditions when different calanoid copepods were fed by a single or mixed diet including various diatom species: *Phaeodactylum tricornutum* [6, 16, 32], *Chaetoceros curvisetum* [16], *C. difficilis* [56], *Cylindrotheca closterium* (= *Nitzchia closterium*) [17], *Ditylum brightwellii* [56], *Pseudonitzschia delicatissima* [38, 50], *Skeletonema costatum* [17,

38], *Thalassiosira rotula* [6, 17, 47], *T. nordenscoldii* [52], *T. weisflogii* [56].

A decrease in viability of eggs produced by copepods fed on diatoms was supposed to result from a deficiency in essential biochemical components [21]. However, analogous anomalies in embryo development were related not only to consumption of diatoms in the field and laboratory conditions, but also to the action of water-soluble diatom extracts applied to the newly spawned eggs of copepods [47]. Originating from diatoms, chemical toxins induce various disturbances in oogenesis and embryogenesis by the blockage of cell division [16, 32, 47, 48]. Antimitotic activity of compounds originating from free fatty acids released during oxidation of lipid components from diatom cells was first reported in 1972 [41]. Recent research found out that antiproliferative action of diatoms is associated with low molecular weight aldehydes [38]. Purified from diatom extracts, low molecular aldehydes produced from different diatoms were identified as 10-carbon aldehydes - decatrienal and decadienal for *T. rotula* [38] and 8-carbon aldehydes - octadienal and octatrienals for *S. costatum* [39]. The degree of antiproliferative effect of purified aldehydes was found to be dose-dependent similar to *the* diatom feeding effect [6, 47].

The mechanism of aldehyde production, considered to be a wound-activated chemical defense from diatoms, was defined as a cascade of sequential chemical reactions [45]. Immediately after disruption of *T. rotula*, phopholipase A_2 generates production of high local concentrations of unsaturated aldehydes from free eicosanoic acids released from *the* diatom cell phospholipids through the programmed mechanism of the typical lipid peroxidation process - aldehyde generating lipase/ lipoxygenase/hydroperoxide lyase cascade. Different diatom species and even strains [45] produce different reactive oxygen species (ROS) but the inhibitory effect was supposed to depend on a reactive structural element – α, β, γ, δ - unsaturated aldehyde [45] produced from free eicosapentaenoic acid (EPA, 20:5n-3) released from disrupted cells.

However, the mode of action of diatoms inhibiting cell division is only hypothesized. Even the data provided by the same authors could be controversial, being either *pro* negative impact of diatoms on copepod recruitment [52], or *contra* this postulate [18]. Until now, it is not clear why various copepod species demonstrate different resistance to the same concentrations of deleterious diatoms. Moreover, inhibition of normal oogenesis is reversible in one species, while is likely irreversible in another [32, 39].

The objective of the present study was to assess and compare the effect of an experimental deleterious diatom diet, and its residual duration after a shift to a positive diet, on a species-specific reproductive response of different but related calanoid copepod species co-occurring in the same natural environment. Our aim was to find out a possible explanation of modifications in diatom-induced embryonic anomalies on the basis of own experimental as well as literary data.

Two major Calanidae species from the Atlantic Ocean, *Calanus helgolandicus*, an indicator of the North Atlantic Central Water, and *Calanoides carinatus,* an indicator of the South Atlantic Central Water [46], were considered to be the appropriate experimental models. These related species co-occur in the N.W. African upwelling area [13] and in the English Channel [20]. The last is the central area of distribution of *C. helgolandicus* and the approximate northern limit of distribution of *C. carinatus*. Both species are fine-particle filter-feeders, predominantly herbivorous, associated with phytoplankton blooms, have generalized life cycles and morphological similarity of larval stages, and do not need re-mating for production of fertilized eggs which develop to hatching in about 1 day [13, 31].

As during the mid-autumn period the food source in the coastal waters in the Western English Channel is scarce, it was considered that experimental diet effect on copepod reproductive response could not be obscured significantly by

feeding *in situ*. Both diatoms and dinoflagellates encountered as typical components of the natural environment in the English Channel and typical diet components for *C. helgolandicus* [11, 28, 31] and *C. carinatus* [13].

Differences in the diatom induced reproductive response of two related copepod species from the same habitat were never tested before simultaneously. Neither was the deleterious effect of diatom diet ever tested on *C.carinatus.* For the first time the same diatom maternal diet (*T. rotula,* actively consumed by copepods and known for its strong deleterious effect [17, 32, 48]) was proposed for simultaneous testing and comparison of the norm of negative reproductive response of *C.helgolandicus* and *C.carinatus*, related species from the same habitat. A dinoflagellate, *P.minimum*, which usually ensures the highest egg viability in copepods [32, 48], was selected to test the effect of positive maternal diet on recovery of reproduction in both species.

Material and Methods. Mature females of *C. helgolandicus* and *C. carinatus* were sorted within 2 h after delivery from the same zooplankton catch (gently towing a 500 μm mesh plankton net obliquely from 20 to 0 m) offshore from Roscoff 48° 45' N and 3° 58' W, in the Western English Channel in October 2001. Females (6 replicates each species) were incubated individually in 150 ml of natural seawater (in 300 ml dishes) and daily transferred to the new dishes with fresh medium. In the first 24 h after delivery (day zero - D0), copepods were incubated in ambient water (filtered through 50-μm) to assess initial fecundity *in situ.* During the following 48 h (D1-D2) copepods were transferred to a fresh suspension of diatoms (*T. rotula*, THA, $5x10^4$ cells per ml determined by microscopic count) in FSW, simulating feeding under short-term diatom bloom conditions to achieve quick reproduction response of both copepod species. Thereafter, copepods were food-deprived for 48 h (D3 - D4) to remove diatom residuals from the maternal organism. From day 5 (D5) onwards, copepod females were switched to a suspension of dinoflagellate *P. minimum,* PRO, $5x10^4$ cells per ml. Microalgae for experiments were used in the exponential phase of growth. Copepods were kept in dim-light conditions at 14±1°C, corresponding to the ambient field temperatures optimal for reproduction of both species [14, 32]. To reduce possible underestimation of (1) egg production (EP, eggs.female^{-1}.d^{-1}) resulting from any injury (cannibalism, or quick autolysis of abnormal eggs during early embryogenesis, etc.), EP was recorded every 4h, except at night, and eggs were transferred for incubation to culture dishes.

Energy resources and essential compounds stockpiled in the oocyte should self-sufficiently support all developmental transitions from the fertilized egg (i.e. cleavage, compaction, blastocyst formation and gastrulation) till hatching of the first larval stage (N1) and its molting into the "first-feeding" larva - nauplii N2. To determine (2) egg hatching success (%H, percentage of hatched eggs from the total number of eggs) and (3) viability of hatched nauplii (%Viab, percentage of N1, molted to N2, from the total number of eggs) batches of 10 freshly spawned eggs were incubated during (2) 24 h – (3) 48 h each in 2 ml of FSW at 14±1°C. Data were statistically analyzed and presented in figures as means and their standard deviations. Copepod late oocytes, embryos and nauplii were checked for morphological features and pigmentation patterns according to visible structures, distinguished under the light microscopy, on specific copepod development stages, as in Poulet et al. [48]: (1) zygote (1 blastomere - 1B); (2) first cleavage (2 blastomeres: 2B); (3) 8-cell stage (8B); (4) morula stage (16B); (5) blastula stage (32B); (6) first naupliar stage N1; (7) second nauplii stage N2. Embryos obtained in experiments were compared with the normal ones described in literature. Normal intact newly spawned eggs of *C. helgolandicus* are characterized by transparent uniform cytoplasm and transparent double membrane ECM, as described by Poulet et al. [48]. Normal eggs of *C. carinatus* present homogenous dark pigmentation of cytoplasm and transparent double membrane ECM,

"covered with branching wrinkles" described by Hirche [13]. During the cleavage of normal embryo, blastomeres are similar, symmetrically organized and present well developed cell membrane between them.

Images were observed and digitized in bright field Nomarsky under the Olympus BH2 microscope equipped with a colour videocamera EuroCam Spot linked to a PC computer, using objectives: Splan 10 0.3 160/0.17 + Splan 20 PL 0.46 160/0.17 and magnification 1.25X +(KOLCO) 0.76X.

Results. Adult females of both species (*C.carinatus,* 2,48±0,03 mm, *C.helgolandicus* - 3,14±0,06 mm) from *in situ* failed to produce eggs without pre-feeding (D0, Fig.1A). Copepods started to spawn after 24 hours of diatom feeding (Fig.1). Egg production of *C. helgolandicus* (EP=10±1 eggs.fem^{-1}.day^{-1}), non-significantly differing from *C. carinatus* (EP=7±2,6 eggs.fem^{-1}.day^{-1}), resulted from diatom (THA) feeding (D1, Fig.A), was typical for copepod late autumn populations. Still, all eggs spawned on D1 by both Calanidae species were abnormal with species-specific differences in type and degree of embryo anomalies (Fig.2A-B). Eggs spawned by C.helgolandicus (D1), presented abnormal stratified blebbed membranes of extracellular matrix (ECM) and cytoplasm with irregular globular structures (Fig. 2A). They autolyzed within several hours at time-interval corresponding to morula-blastula stage in normal embryos, and resulted in 0 % hatching (Fig.1B).

Eggs produced by *C. carinatus* (D1) were characterized by normal ECM and relatively synchronous cleavage, presenting dark-brown pigmentation accented in proximities of cell membranes, particularly, within the cleavage furrows of dividing blastomeres, that is clearly distinguished by light microscopy on the morula stage (Fig. 2B). Egg hatching success was 100 % (Fig.1B) but all nauplii N1 revealed typical diatom-induced morphological deformities as is described in [48], i.e. asymmetrical development of the body, more reinforced on the right side, abnormally shortened and thickened appendages developed asymmetrically, resulted in crumpled movements of the nauplii, with asymmetrical distribution of brownish pigment granules in the body and appendages (Fig 3A). All nauplii N 1 died and did not molt into the N 2 stage. Thus, viability was 0 (Fig.1C).

Spawning continued during the second day of THA feeding (D2), with EP differed non-significantly between two species (17,7±3,1 and 22,7±8,6 eggs.fem^{-1}. day^{-1}, for *C.helgolandicus* and *C. carinatus,* respectively; Fig.1A), but egg hatching success diminished to 0 for both species (Fig.1B). The anomalies of Calanidae spp. eggs increased in variability and degree of embryo degeneration. General patterns for D2 embryos were severe degeneration of ECM membranes, asymmetry of cytoplasm structures and erroneous distribution of pigment granules in cytoplasm. Degree of anomalies varied between species and between different specimens within one species**.**

A small portion of *C. carinatus* eggs presented thick condensed (lacking transparency) blebbed membranes of ECM. They stopped development at the late embryo stage. Distinct asymmetry in the body and limbs and erroneous distribution of abnormal brown pigment granules in the embryo cytoplasm were observed (Fig. 2C).

The majority of the eggs spawned by females of both species on D2 displayed disintegration of thin outer and inner membranes of ECM lacking hyaline layer between. Half of these eggs underwent relatively synchronized cleavage until 16B (*C. carinatus,* Fig. 2E), or 32B (*C. helgolandicus*, Fig. 2D) stage, and after 4-5 mitotic divisions development stopped. Blastomeres of these eggs did not *flatten* in the absence of normal cell adhesion at cell membranes, and, as a result, compaction did not occur. The embryos, instead of formation of normal morula, presented a "grape-like" group of round blastomeres disposed asymmetrically within the thin membrane. The other part of the eggs, with even more degenerated membranes, stopped development during

the first cell division and presented pigment granules either erroneously distributed within the blebbed cytoplasm in *C. carinatus* (Fig.2F) and *C.helgolandicus* (Fig.2G), or concentrated at the poles of the polarized zygote (Fig.2H), or localized in the proximities of the first cleavage furrow (*C. carinatus,* Fig.2I)**.**

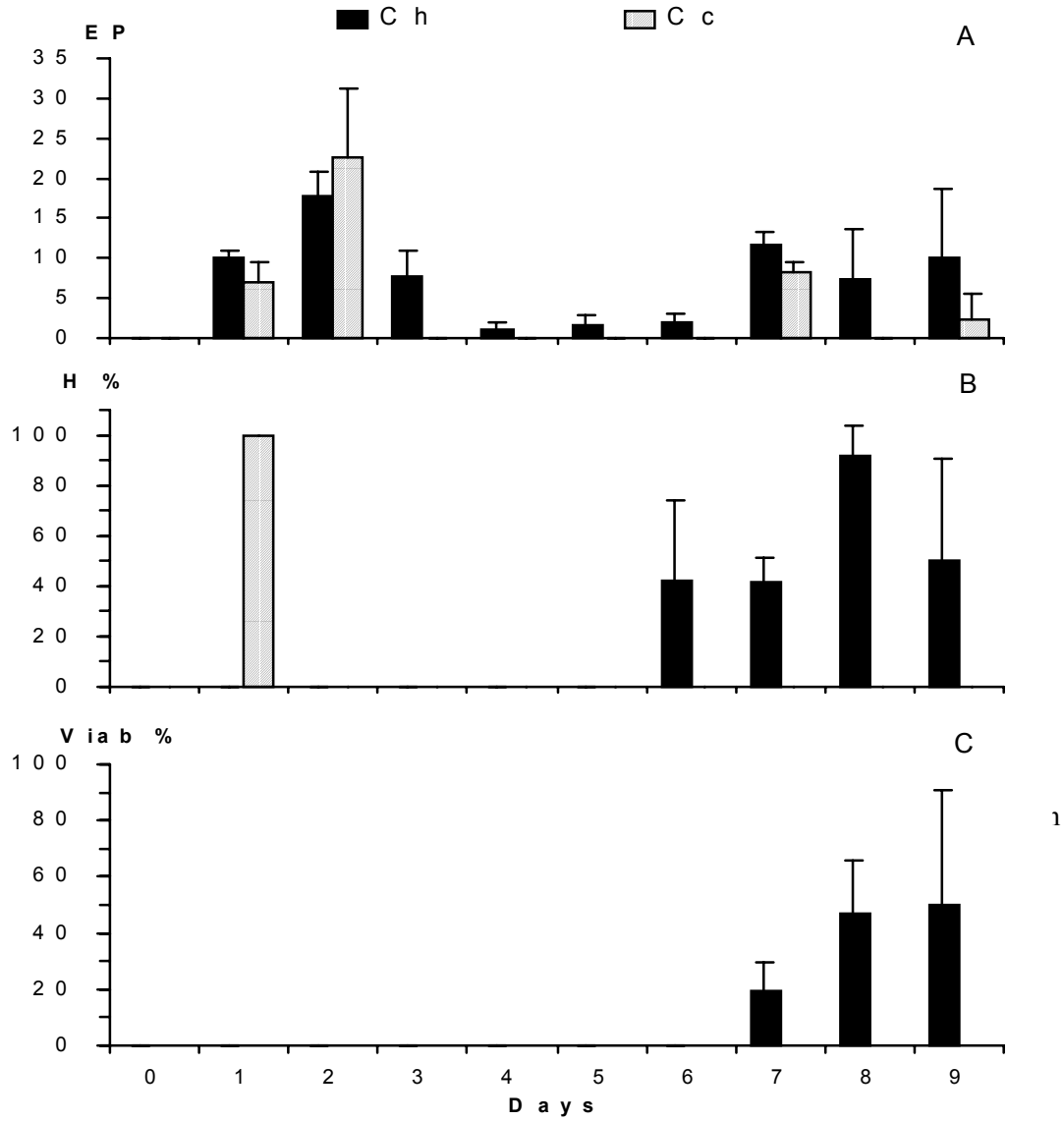

Fig.1. Egg production (EP, eggs.day^{-1}.female^{-1}) (A); hatching success (H %, percent of hatched eggs of the total number of spawned eggs) (B); viability % (Viab %, per cent of viable nauplii I of the total number of spawned eggs) (C) of *Calanus helgolandicus* (Ch) and *Calanoides carinatus* (Cc) during experimental feeding: *in situ* - D 0; *Thalassiosira rotula* - D 1-2; starvation - D 3-4; *Prorocentrum minimum* - D 5-9 . Values (means) and error bars (standard deviations) of 6 replicates.

Рис.1. А. Продукция яиц (EP, яиц.сут$^{-1}$.самка$^{-1}$) (A); процент выклева (H %, процент выклюнувшихся яиц к общему числу отложенных яиц) (B); жизнеспособность (Viab %, процент жизнеспособных науплиев к общему числу отложенных яиц) (C) *Calanus helgolandicus* (Ch) и *Calanoides carinatus* (Cc) при питании: *in situ* D0; *Thalassiosira rotula* - D 1-2; голодание - D 3-4; *Prorocentrum minimum* - D 5-9. Приведены средние значения величин и их стандартные отклонения (6 повторностей)

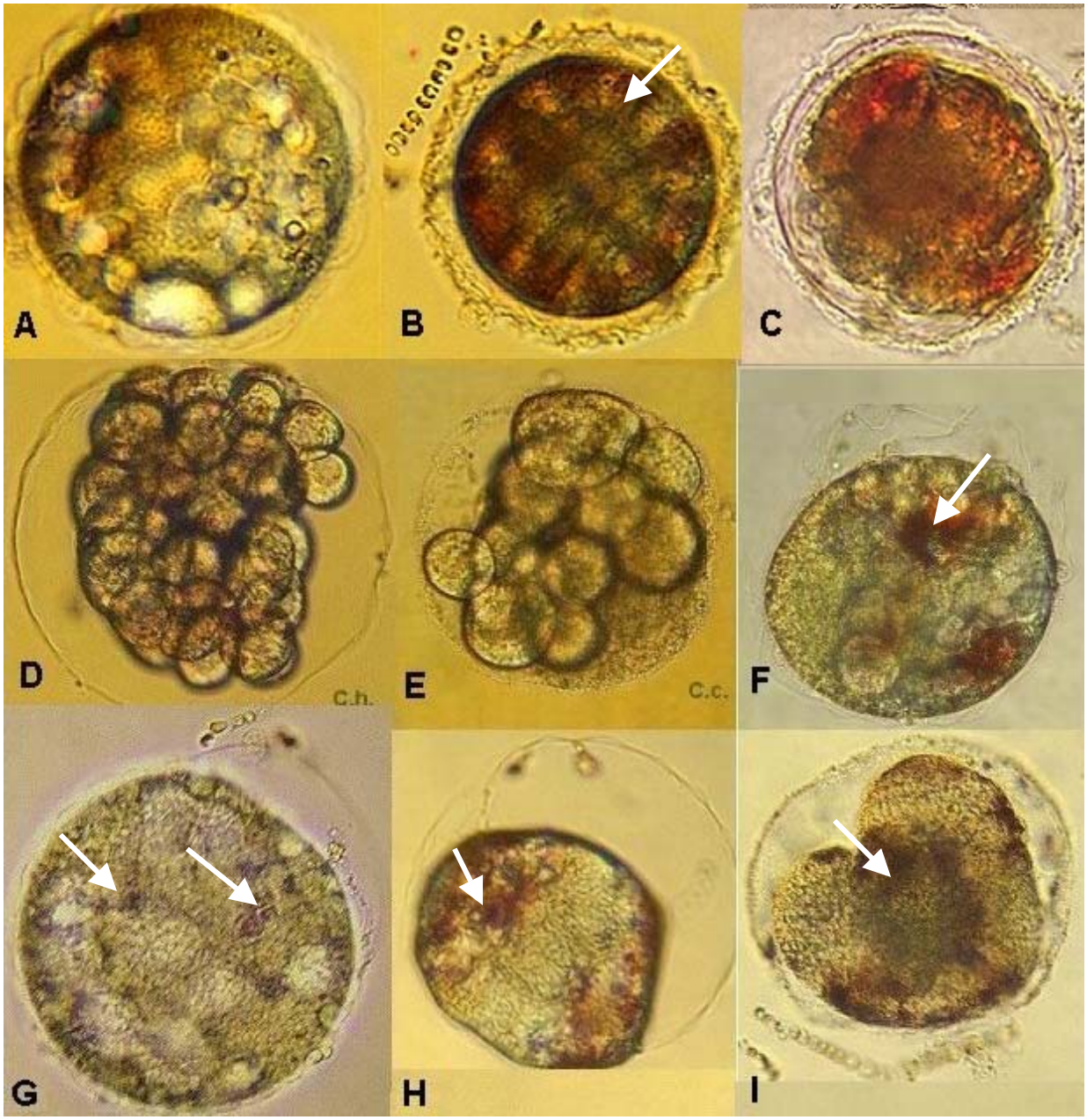

Fig. 2. Types of embryo anomalies in *C. helgolandicus* (A, D, G) and *C. carinatus* (B, C, E, F, H, I) resulted from diatom *Thalassiosira rotula* "maternal diet" on D1 (A, B, C) and on D2: (D, E, F, G, H, I). Only type B developed till hatching into abnormal nauplii I (Fig.3A). Arrows show abnormal dark-brown pigment distribution in eggs.

Рис. 2. Типы аномалий эмбрионов *C. helgolandicus* (A, D, G) и *C. carinatus* (B,C, E, F, H, I) на первые - D1 - (A, B, C) и вторые сутки - D2:- (D, E, F, G, H, I) при питании самок диатомовыми *Thalassiosira rotula.* Развитие до науплия I (аномального, рис.3A) происходит только из яйца типа B. Стрелки указывают на аномальное распределение темно-коричневого пигмента в яйцах.

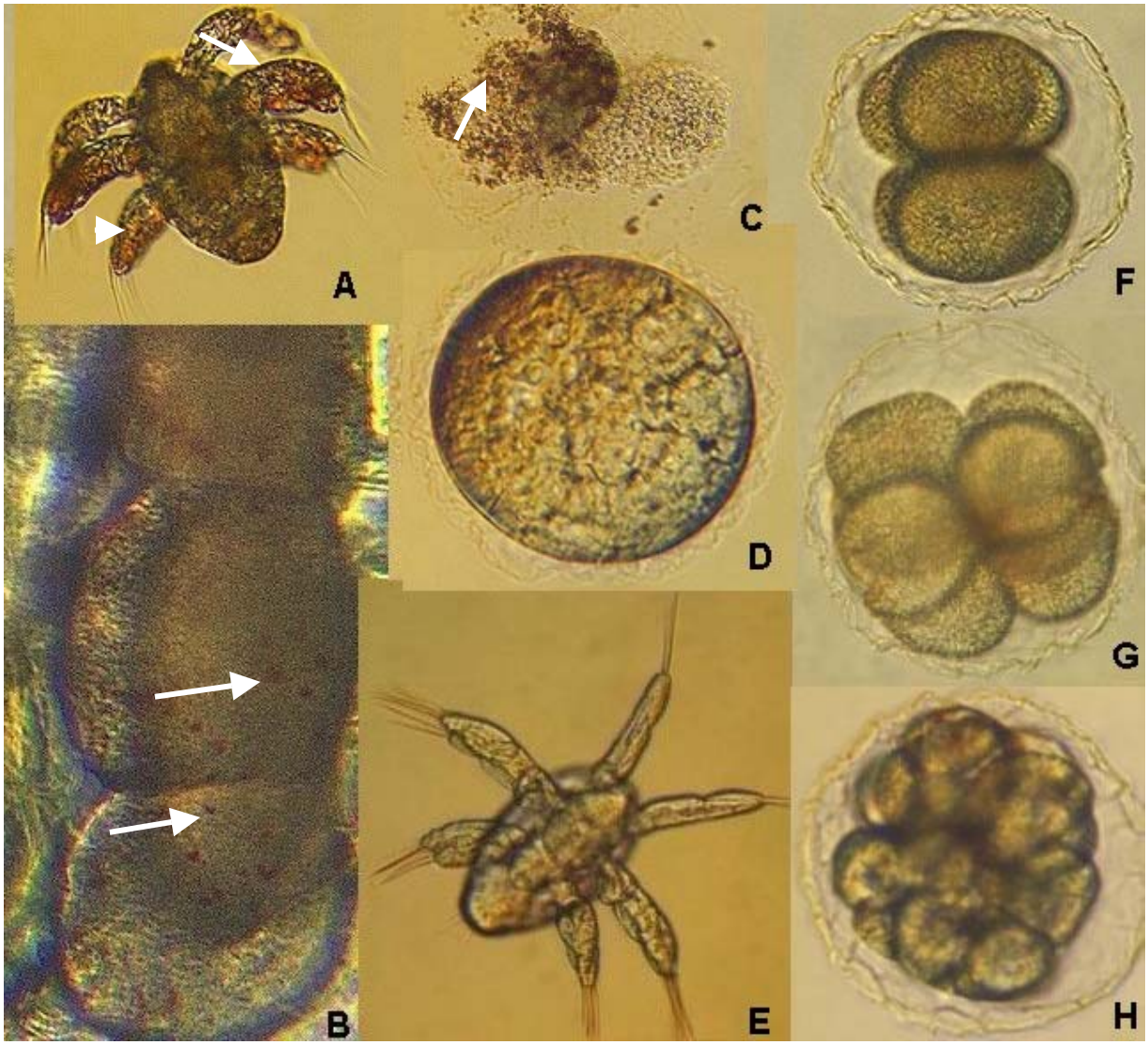

Fig. 3. Residual effect of *Thalassiosira rotula* “maternal diet”:
(A) Abnormal nauplii N I resulted from *C. carinatus* abnormal egg (Fig.2 C).
(B) Abnormal pigmentation in the late oocytes in the oviducts of *C. carinatus* fed *T.rotula*
(C) "Marine snow" formation from abnormal embryo
(D) Viable egg (20 %) of *C. helgolandicus* fed *P.minimum* (D7)
(E) Normal nauplii N I *C. helgolandicus* resulted from the egg Fig.3D
(F-G) Cleavage of abnormal embryo from *C. carinatus* female after feeding *P.minimum* (D8)
Arrows show dark-brown pigment granules

Рис. 3. Остаточный эффект "материнской"диеты *Thalassiosira rotula:*
(A) Аномальный науплий N I *C. carinatus*, полученный из аномального яйца (рис. 2 C).
(B) Аномальная пигментация поздних ооцитов в яичниках *C. carinatus* после питания *T.rotula*
(C) Формирование "морского снега" при лизисе аномального эмбриона
(D) Нормальный эмбрион *C. helgolandicus* после питания динофлагеллятами *P.minimum* (D7)
(E) Нормальный науплий N I *C. helgolandicus,* полученный из яйца (рис. 3 D)
(F-G) Дробление аномального эмбриона *C. carinatus* после питания *P.minimum* (D8)
Стрелки указывают на темно-коричневые гранулы

Eggs with severe deterioration often accumulated the gases between the plasma and outer membranes and floated to the surface (while normal eggs sink), where they soon "burst" into the substance similar to "marine snow" (Fig.3C). Microscopic observations *in vitro* revealed that free-living “run-and-tumble”-swimming heterotrophic bacteria, typical for “marine snow” aggregates, attracted chemokinetically by organic solutes [33] leaking out from disrupted abnormal eggs, were observed colonizing the scattered layers of outer membranes within several minutes, destroying them actively, and penetrating through inner membrane into the embryo cytoplasm within 30 minutes.

Females of both species changed gradually initial species-specific "wild pigmentation" patterns during 2 days of THA feeding. *C. helgolandicus* totally lost the rose-pink coloration, and intensive red pigmentation of *C.carinatus* was also significantly reduced. Erroneously distributed pigment granules in the late oocytes were easily detected in *C.carinatus* female oviducts through transparent integuments under the light microscope on D3 (Fig.3B).

During starvation in FSW (D3-D4) *C.carinatus* totally ceased spawning, meanwhile, *C.helgolandicus* reduced gradually the production from 7,2 to 1 eggs.fem^{-1}. day^{-1} (Fig.1A) of abnormal eggs, lacking outer membrane and disrupting prior the start of cleavage.

After switching to the PRO diet, *C. helgolandicus* resumed egg production (D7) (Fig.1A) to the level typical for autumn populations of this species [32], but only about 20 % of spawned eggs displayed normal membranes (Fig.3D). These underwent typical cleavage and hatched (Fig.1B) into normal viable nauplii N1 (Fig.3E). Viability increased up to 50 % on D9, coinciding with hatching success (Fig.1B, C).

C. carinatus fed PRO also resumed pulsatory egg production (Fig.1A), but hatching success was 0 % (Fig.1B). These eggs spawned D7-D9 still presented anomalies resulting from the D1-D2 diatom maternal diet (Fig.3F). The ECM of these eggs were similar to the ECM of normal eggs (inner and outer membranes with hyaline layer). The cytoplasm was uniformly pigmented, but blastomeres displayed reduced adhesion and compaction did not occur. The majority of the embryos stopped development after 3 synchronous cell divisions at 8B stage (Fig.3G). The rest never survived after the 16-cell stage (Fig.3H). None of eggs produced by *C. carinatus* females resulted in viable nauplii during the 10 days of the experimental period.

Thus, it was observed that experimental “diatom bloom” conditions resulted in total mortality of the progenies of both copepod species. *C. helgolandicus* showed quick reproductive responses: both negative on diatoms, and positive when switched to dinoflagellates (recovered quickly the egg production and viability). Both reproductive responses of *C. carinatus* were postponed, and the residual negative effect of diatoms on embryos was observed during the experimental period of dinoflagellate feeding.

Discussion. The state of cell membranes as one of the criteria for identification of anomalies in the eggs of *C. helgolandicus* was discussed in Poulet et al. [48]. Abnormal eggs were usually characterized either by "dark brown, opaque" [47] or "darker and more granular" [48] outer membranes in comparison with that of normal eggs in early stages of development (zygote - 2 cells), or presented “malformations of the cellular membrane between daughter cells during mitosis”, or even “the absence of a cell membrane between daughter cells" at 16- (morula) -32-cell (blastula) stage embryos.

Earlier, Hirche [13] described abnormal eggs of *C. carinatus* from Cape Bojador "without space between inner and outer membranes" with the "substance ... divided into many smaller spheres" (similar to Fig. 2E, present paper) attributed by the author to "decrease of supply of viable sperm". Still, these anomalies could be more reasonably supposed to be *also* related also to "maternal diet effect", as, according to the same author (Table 2, 13]), 100 % egg hatching success re-

sulted from *C.carinatus* fed dinoflagellate (*Gymnodinium* sp.) diet in comparison with 75 % hatching success of eggs from females fed diatom (*P. tricornutum*).

We ranked diatom-induced anomalies observed during Calanidae embryogenesis (our own and literature data) by a bottom-up principle, arranging them according to increase of degree of embryo degradation (**DED**) identified by microscopic observations and related to the stage of embryo death:

DED 1. Eggs display a normal transparent multilayer extracellular matrix (ECM), and undergo normal cleavage, compaction and gastrulation. Still, slight deterioration is observed in pigmentation, emphasized in the proximities of membranes (in cleavage furrows) of dividing cells (Example: *C. carinatus*, Fig.3B, present paper). Development of such eggs result in hatching of abnormal nauplii N1 with shortened massive bodies flexed dorso-ventrally; asymmetrical*ly*, shortened, abnormal*ly* in shape and segmentation appendages, antennule, antenna and mandible with atypical reduced number and length of bristles and darker opaque body colour with erroneous distribution of brownish pigment granules (Examples: *C. helgolandicus:* Fig.4F, Fig.5C-D [48]; *C.finmarchicus*, Fig.4C [52]; *C.euxinus*, own data, unpubl.; *C. carinatus*: Fig.3A, present paper). Development is arrested at stage nauplii I.

DED 2. Eggs display multilayer but non-transparent (opaque) thick ECM with blebbed membranes, undergo synchronized cleavage and gastrulation, but the late embryo displays severe asymmetrical cytoskeleton with abnormal distribution of pigment granules. Development stopped prior to hatching (Examples: *C.helgolandicus,* Fig.4B [48]; *C.finmarchicus*, Fig.4B [52]; *C.euxinus*, our own data, unpubl.; *C.carinatus*, Fig.2C, present paper). Development is arrested at the late embryo stage.

DED 3. Eggs show deterioration (stratification) of the multilayer ECM. The cytoplasm of the embryo is characterised by scattered, irregular, asymmetrical globules corresponding to nuclei in the absence of normally organized cell membranes between dividing cells (probably resulting from deficiency of hyaline to form normal cell membranes). On the borders, the abnormal brownish pigment is concentrated. Karyo- and cytokinesis are desynchronized from the start of cell division (after [48]). (Examples: *C.helgolandicus,* Fig.3E, G [48]; Fig.2A, 2G, present paper; *C.finmarchicus*, Fig.4A [42]; *C.carinatus*, Fig. 2F, present paper). Development is arrested at a time corresponding to the morula stage in normal embryos.

DED 4. Eggs lack a multilayer ECM (transparent outer and inner membrane and hyaline layer between), i.e. "the space between inner and outer membranes" [13], and often display a severely deteriorated thin one-layer outer membrane. Cleavage takes place until the 16-cell or 32-cell stage. Cell adhesion is absent (presumably, the cell membranes lack tight junctions as a result of *the* absence of a hyaline layer or deformation of microtubules or both) and blastomeres do not flatten ("the substance is divided into many smaller spheres" [13]. (Examples: *C.carinatus*, Fig.3B, [13]; Fig.2E, present paper; *C.helgolandicus*, Fig.2D, present paper; *C. euxinus*, our own data, unpubl.). Compaction does not take place, the morula does not develop. Development is arrested during cleavage, after 4 - 5 cell divisions.

DED 5. (diatom residual, observed during recovery diet, intermediate). Eggs present a multilayer ECM, undergo synchronized doubling of cell numbers until the 8-cell (rarely 16-cell) stage, cell adhesion is reduced, and blastomeres do not flatten. (Example: *C.carinatus*, present paper, Fig. 3F-H; C). Development is arrested after 3 - 4 divisions (prior to cell polarization).

DED 6. Eggs display a high degree of cytoplasm and ECM deterioration with stratification of membrane from the cytoplasm. Abnormally scattered pigment granules are disposed on different poles of the 1-cell embryo (zygote), or concentrated near the developing first cleavage furrow. (Examples: *C.carinatus*, Fig.2H-I, present paper). Development is arrested prior to the 2B stage.

DED 7. Late oocytes resorb, presumably lacking normal cortical granules and membranes during late vitellogenesis. Developmental arrest occurs at the late oocyte stage. (Example: *C.helgolandicus,* [32]; present study; *C.carinatus*, present study; *C. euxinus*, our data, unpubl.).

On the basis of this ranking it was concluded that the common patterns in all diatom-induced embryo anomalies are accumulated-dose deteriorations in extracellular matrix (ECM) and pigment granule distribution.

Role of the ECM in coordination of cell stability. Many cellular events of embryo development during cleavage and, especially, at and after gastrulation, i.e. morphogenesis, cell migration, shape change, proliferation and gene expression, are dependent on interactions with the ECM environment. The major constituent of the embryo ECM is the calcium-ion-dependent hyaline layer, functioning as a substrate for cell adhesion through early development up to start of gastrulation [57]. The major constituent of the hyaline layer, the protein hyaline, multimerizing in the presence of calcium, is very important for blastomere adhesion and for repacking of cells during the gastrulation, invagination and formation of gut and lumen. All present in the embryo hyaline protein is maternally derived from the original cortical granules exocytozed at fertilization and remains at relatively constant levels throughout embryo development. Thereafter, it is assumed we assume that all the above-ranked diatom-induced malformations of nauplii and eggs (DED 1-7) are expressed in successive reductions of ECM related to diatom-dose-dependent decrease of hyaline protein, possibly related to cortical layer anomalies.

Role of pigment in the coordination of cell stability. Pigment granules are important components of the cortical layer. As the main vesicular component of egg cortices perfused with calcium, they provoke cortical granule exocytosis at fertilization [55], governing the formation of the hyaline layer. In a normal intact embryo, blastomeres should be structurally polarized so that all microvilli and cortical "pigment granules" are situated at the apical surfaces facing the hyaline layer and are absent from basolateral surfaces facing adjacent blastomeres and the internal embryonic cavity. Our observations of scattered pigment distribution in abnormal early embryos (Fig.2A, 2F, 2G, 2H-I) and cryptic late embryos (Fig.2C) and nauplii I (Fig.3A) could be undoubtedly ascribed to gradual changes in pigment contents in females during diatom feeding. Irregularly distributed abnormal brownish pigment granules in *C.carinatus* late oocytes (Fig. 3B) could be also attributed to inadequate pigment (carotenoid) content of the diet. Convertible calcium-carotenoid transformation is very significant in regulation of lipid peroxidation during embryogenesis [57]. Carotenoid containing parts of mitochondrias are specialized in convertible accumulation of calcium salts [37]. Carotenoid imbalance can lead to calcium ion deficiency, cause membrane `blebbing' and shedding of plasma membrane vesicles which lack cortical cytoskeleton and are deficient in cytoskeleton proteins and devoid of microvilli [60], thus reducing cell adhesion and leading to dissociation of blastoderm into separate blastomeres, as was observed in abnormal embryos in our experiments (Fig. 2D-I).

The explanation of deleterious action of unsaturated aggressive aldehydes from diatoms does not completely explain the ECM and pigment deteriorations in embryos and total cessation of egg production, as similar features of egg pathologies were observed with non-diatom diets, never reported for production of toxic aldehydes. Earlier (our own experimental data 1999-2000, unpubl.), we observed similar effects in the eggs of *C.helgolandicus* spawned after feeding cryptomonads, *Rhodomonas salina* [25] and *R. baltica* [26]. Eggs often presented abnormal deep-pink pigmentation coupling with abnormal morphological patterns, i.e. reduced ECM and absence of adhesion between blastomeres, and cytolyzed at the 8-cell stage (similar to Fig. 3F-H). *C.helgolandicus* females fed on the green microalgae, *Dunaliella tertiolecta,* also not only quickly displayed reduced viability of spawned eggs but

totally stopped egg production as a result of irreversible atresia of the late oocytes, attributed to limitation of essential nutrients in food, specifically highly unsaturated fatty acids, HUFA [32]. Such failures of copepod reproduction indirectly confirm that anomalies in copepod eggs are likely caused by inadequate lipid and pigment contents and/or synthesis in the embryo following inadequate composition and ratio of these essential constituents in the maternal diet.

Role and Synergism of essential constituents in embryo development. Any anomalies in development leading to the death of the embryo or nauplii I reflect disturbances of embryo homeostasis, that is, disorder in its metabolism, often revealed in fatty acids imbalance [1]. The primary role in copepod embryogenesis is attributed to lipids as oxidative fuels that support embryo growth and also as components of the newly formed cell membranes. Functionality and integrity of cell membranes rely significantly on physical properties of membrane phospholipids determined by their fatty acid composition, specifically, high HUFA, specifically, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) contents and ratio. The ratio of DHA to EPA affects membrane permeability, fluidity, membrane-bound enzymes [59], proteins residing in the membrane, and gene expression. It is likely that antioxidant protection in crustaceans, and specifically in calanoid copepods and their food web, normally involve coupling of these HUFA with lesser essential constituents, specific carotenoids scavenging free radicals [29, 33, 36]. Copepods display a certain linkage of their fatty acid composition and degree of oxygenation of their carotenoids [12, 36].

Lipid composition of calanoid copepods is characterized by high contents of EPA and DHA (sum ranging 50-80 % from total fatty acids [7, 24, 35, 59]) with the DHA/EPA ratio varying from 0,9 to 6,1 [7, 24, 35, 59]. Main dominating carotenoid, active in copepod lipid metabolism is astaxanthin, ranging from 60 to 87 % of all copepod pigments [33, 35]. Astaxanthin is found as the main pigment occurring in the vitellus of copepod eggs [30], and rapid lipid metabolism during early development coincides with the astaxanthin esterification, principally by EPA and DHA [36], undergoing concomitantly with the resorption of vitellin resources [5]. Synergy of the DHA/EPA content and astaxanthin level in phospholipids was considered as one of the most important determinants of the egg quality in marine organisms [43], as it affects the structure of cytoplasm, and specifically, the structure and physiological properties of egg membranes [12]. Indeed, astaxanthin maintains structural stability of easily oxidized lipid components, particularly HUFA [30, 36] inhibiting lipid peroxidation in biomembrane systems. The strongest antioxidant properties of astaxanthin against any oxidative stress damaging cellular lipids, proteins, and DNA are specified due to its particular structure and specific location in the phospholipid membrane [10, 36]. Scavenging free radical chain reactions both at the surface and in the interior of the phospholipid membrane in a dose-dependent mode [10], it promotes ion balance, cell adhesion, differentiation and growth control, therefore, normal homeostasis and morphogenesis in the embryo [37].

Effect of diet on copepod essential constituent composition and egg viability. Both essential components, HUFAs and carotenoids, could not be synthesized *de novo* by calanoid copepods but rely on their content in consumed food [1], i.e. on diet microalgae composition. Absorption of fatty acids and carotenoids from food particles is dependent on the same mechanism of emulsification by bile salts, and both essential components are transported in hemolymph in the conjugated form [12] wherefrom they are likely non-selectively endocytosed (by pinocytosis) by the late oocytes [47]. Metabolic pathways of certain fatty acids and carotenoids from microalgae in copepods are still not specified but it is likely that calanoids differ from other crustaceans by their inability to metabolize astaxanthin directly from beta-carotene, as well as DHA directly from its diet precursors.

The dinoflagellate *P. minimum* (positively correlating with the highest viability of copepod eggs) differs from the diatom *T. rotula* (negatively correlating with the lowest egg viability) in the ratio of polyunsaturated to saturated fatty acids (3.09 *versus* 0.94) and ascorbic acids (45.6 *versus* 17.3) (calculated from [15]). In dinoflagellates, *P. minimum* included [1], DHA / EPA ratio is always higher than 1 [9, 49, 53, 61]. On the contrary, diatom lipids are invariably rich in EPA [9, 49, 61] with DHA/EPA ratio as low as 0.02 - 0.22 in comparison with dinoflagellates and cryptomonads (0.50 -0.63). In particular, in diatom *T. rotula* EPA and DHA constitute, respectively, 30,5 % and 5,4 %, of the total fatty acids (Kattner G., unpubl.). Carotenoid content of these microalgae groups *also* as well differs significantly: fucoxanthin dominates in diatoms, alloxanthin – in cryptomonads (both lack from dinoflagellates), and dinoflagellate carotenoids are dominated by peridinin [8, 44] found among these the most efficient quencher of free radicals [19].

Dietary fatty acids are incorporated by adult copepods practically unchanged [9]: fed diatoms they increase (and with cryptomonad diet less rich) in EPA [9, 54], whereas fed dinoflagellates, they increase DHA [24, 49, 54]. Carotenoid content of calanoid copepods also depends significantly on feeding [29; our own results, unpubl.], and can double within 24 h through the diet effect [22].

The fatty acid profile of copepod eggs and newly hatched nauplii I, as well, reflect the fatty acid composition of the maternal microalgal diet. The diatom maternal diet is coupled with a EPA prevailing DHA in copepod eggs [32] and newly hatched nauplii [54], while in eggs and nauplii originated from the dinoflagellate diet, DHA prevails over EPA [32, 54]. The viability of calanoid eggs was found positively correlated with the ratios of polyunsaturated to saturated, n-3 to n-6 and DHA/EPA fatty acids among the total fatty acids in the maternal phytoplankton diet [15, 53] and DHA/EPA ratios in eggs [32, 50] and nauplii [54]. An imbalance in a carotenoid to fatty acid ratio in eggs is as well connected with deterioration in eggs development [5, 43]. In addition, astaxanthin in copepods was found to decrease significantly when phytoplankton was dominated by diatoms [2].

We assume that as both the HUFA and astaxanthin content and ratio in copepods depend significantly on the maternal diet supply, the normal development of the copepod embryo requires a specific content and ratio of HUFA coupled with specific content of carotenoid (close precursor of astaxanthin) to be present in microalgae diet. Obviously, EPA content in freshly assimilated food determines the level of egg production, being catabolized as an energy supply for egg production and embryo development, while DHA and astaxanthin determine the egg viability, being selectively incorporated into cell membranes.

Lipid peroxidation mode of diatom action on recruitment of Calanoidae is proposed. Excess of EPA is known to increase fatty acid oxidation in mitochondria [34], while astaxanthin exhibits the highest among carotenoids inhibitory effect on mitochondrial lipid peroxidation. At the bottom of diatom-induced gradual reduction of ECM, mitosis disorder and abnormal pigment distribution in the embryos and oocytes is supposed to be the increase of lipid peroxidation in mitochondria and cell membranes of the copepod embryo (caused by excess of EPA) coupled with reduction of radical scavenging activity of membrane phospholipids (caused by decrease of astaxanthin). Aggressive low molecular aldehydes generated from disrupted diatom cell [45] provoke extra oxidative stress.

Peroxidation of HUFAs is particularly deleterious for embryo. High concentration of ROS impair phospholipid biomembranes of the embryo, damage lipid and protein key molecules, cause DNA fragmentation [40] and as a consequence, lead to embryonic dysmorphogenesis. Oxidative stress can arise mutagenesis of the maternal mRNAs as well as changes in translation of mRNAs [55, 57] synthesized during diatom-induced oocyte growth and result in deterioration of hyalin- and pigment-containing cortical granule

organization, and thus affect not only the earliest development changes (deficiency of hyaline substrate for adhesion), but mRNA functioning during late development, e.g. during gastrulation, causing the pathology in the anterior-posterior axis (cryptochordism) of embryo.

Species-specific differences in the degree of diatom-induced embryo anomalies. Involved in egg production in crustaceans, essential HUFA and carotenoids are fuelled from two sources, i.e. internal stock reserves from earlier feeding and freshly assimilated food [12]. The quota of freshly assimilated organics used during vitellogenesis for oocyte maturation could be ascribed to the species-specific differences in metabolic pathways of freshly assimilated organics and to individual feeding prehistory. Typical upwelling species, *C. carinatus*, well adapted to the long-term storage of a large amount of energy and to the long-term food variability [14], presents a typical lag-phase in reproductive response to both "negative" (diatoms) and "positive" (dinoflagellates) diets in comparison with a quick response of *C. helgolandicus,* adapted to well mixed homogenous coastal waters of English Channel [31]. A delayed response indicates the existence in *C.carinatus* of a buffer of nutrients from an earlier assimilated diet and incorporation of more internal stockpiled material from its plentiful lipid reserves [14] sequestered at some earlier point in their life cycle to synthesize eggs [51]. On the contrary, *C.helgolandicus* is likely fuel more egg material from external sources through direct accumulation of freshly assimilated organics, especially its essential components, from the maternal diet. Differences in coloration between the wild specimens of two studied species (slightly rosy in *C. helgolandicus* and intense red in *C. carinatus*) could be related to the differences in their stockpiled carotenoids, acting as antioxidants for lipid storage.

Conclusions. Diatom-induced abnormalities of the copepod embryos concern defects in organization of extracellular matrix and pigment contents. Defects are supposed to be attributed to the disturbances in antioxidant properties of the membranes and the degree of cell lipid peroxidation related to diatom-dose dependent decrease in DHA/EPA ratio and astaxanthin contents in copepod eggs. Species-specific differences in copepod response to diatom feeding are likely attributed to different contributions of essential components from stock-piled essential material, and those derived directly from diet particles, to the late oocytes. Disrupted copepod non-viable eggs and nauplii I during diatom blooms form aggregates of particulate and dissolved organic matter similar to "marine snow". Hypothesis needs further investigations.

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Видоспецифические различия индуцированных диатомовыми аномалий эмбриогенеза каланоидных копепод: связаны ли они с запасными антиоксидантами? А. Н. Ханайченко. В экспериментальных условиях сравнивали продукцию и жизнеспособность яиц от самок двух видов каланоидных копепод – *Calanus helgolandicus* и *Calanoides carinatus* при питании диатомовыми, *Thalassiosira rotula,* и при переходе на питание динофлагеллятами, *Prorocentrum minimum* Самки обоих видов при питании диатомовыми продуцировали сходное количество яиц (до 17-22 яиц·самку$^{-1}$·сут$^{-1}$), но все эмбрионы были нежизнеспособны. Развитие копепод, в зависимости от степени аномалий, останавливалось на разных стадиях эмбриогенеза. У аномальных эмбрионов обнаружены разнообразные отклонения в распределении пигмента и организации экстраклеточного матрикса. Репродуктивные отклики *C.carinatus* как на негативную (диатомовые), так и позитивную (динофлагелляты) диеты отсрочены по сравнению с быстрыми откликами *C. helgolandicus*. Через 3 сут после начала питания динофлагеллятами у аномальных эмбрионов *C. carinatus* наблюдался сильный остаточный эффект диатомовой диеты, в то время как 50 % яиц *C. helgolandicus* были жизнеспособны. Предложена гипотеза, связывающая степень аномалий, вызванных диатомовыми, со снижением антиоксидантных характеристик мембран и степенью перекисного окисления липидов в мембранах и цитоплазме эмбрионов, индуцированных дисбалансом соотношения незаменимых ВНЖК и каротиноидов из свежеассимилированной пищи. Видоспецифические различия репродуктивного отклика на диету объясняются различиями в количестве запасных незаменимых компонентов и путях их метаболизма у разных видов копепод.

Ключевые слова: каланоидные копеподы, диатомовые, эмбрион, аномалии, экстраклеточный матрикс, пигмент

Видоспецифічні розходження індукованих діатомовими аномалій ембріогенезу каланоїдних копепод: чи зв'язані вони із запасними антиоксидантами? А. М. Ханайченко. В експериментальних умовах порівнювали продукцію та життєздатність яєць від самок двох видів каланоїдних копепод *Calanus helgolandicus* і *Calanoides carinatus* при живленні діатомовими мікроводоростями, *Thalassiosira rotula,* і при переході на живлення дінофлагелятами, *Prorocentrum minimum* Самки обох видів при живленні діатомовими продукували подібну кількість яєць (до 17-22 яєць·самку$^{-1}$ ·доб.$^{-1}$), але всі ембріони були нежиттєздатні. Розвиток копепод зупинявся на різних стадіях ембріогенезу залежно від ступеня аномалій. В аномальних ембріонах виявлені

різноманітні відхилення в розподілі пігменту й організації экстраклітинного матріксу. Репродуктивні відгуки *C. carinatus* як на негативну (діатомові), так і позитивну (дінофлагеляти) дієти відстрочені в порівнянні зі швидкими відгуками *C. helgolandicus*. Через 3 доби після початку живлення дінофлагелятами у аномальних ембріонів *C. carinatus* спостерігався сильний залишковий ефект діатомової дієти, у той час як 50 % яєць *C. helgolandicus* були життєздатні. Запропонована гіпотеза, що зв'язує ступінь аномалій, які викликаний діатомовими, зі зниженням антиоксидантних характеристик мембран і ступенем перекісного окислювання ліпідів у мембранах і цитоплазмі ембріонів, індукованих дисбалансом співвідношення незамінних ВНЖК і каротиноідів зі свіжо асимільованої їжі. Видоспецифічні розходження репродуктивного відгуку щодо дієти пояснюються розходженнями в кількості запасних незамінних компонентів і шляхах їхнього метаболізму у різних видів копепод.

Ключові слова: каланоїдні копеподи, діатомові, ембріон, аномалії, экстраклітинний матрикс, пігмент