Oral brooding (*Anasterias antarctica*) versus internal brooding (*Diplopteraster verrucosus*): contrasting strategies of the brooded juveniles for full development

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Abstract: Both sea star species in this study are brooders from the South Atlantic. *Anasterias antarctica* displays a complex reproductive cycle involving incubation during eight or nine months and it broods its offspring over the mouth, while *Diplopteraster verrucosus* displays continuous reproduction and broods its offspring in nidamental chambers under the dermis. We collected *A. antarctica* in May, August, and November (beginning, mid and late incubation) from Parque Nacional Tierra del Fuego (Tierra del Fuego AeIAS, Argentina), while *D. verrucosus* was collected at three Oceanographic Research Cruises at Namuncurá Marine Protected Area/ Burdwood Bank I and II. We measured general parameters of oxidative metabolism (reactive oxygen species "ROS" and total antioxidant capacity "TAC"), energy density, and energy content in three different stages during offspring development. In the case of *A. antarctica*, energy depletion, increasing ROS and constant TAC levels at the late development stage could act as a developmental constraint and trigger the independence of juveniles. Similarly, in *D. verrucosus* juveniles, which exhibit extra embryonic maternal nutrition, despite the continued increase in energy, the antioxidants present in this kind of nutrition do not counteract the ROS generated by the metabolic rise.

Key words: Embryos, sea stars, oxidative metabolism, energy density, South Atlantic

Resumen: Incubación oral (*Anasterias antarctica*) vs. incubación interna (*Diplopteraster verrucosus*): estrategias contrastantes de los juveniles incubados para desarrollarse completamente. En este estudio ambas especies de estrellas de mar son incubadoras del Atlántico Sur. *Anasterias antarctica* posee un ciclo reproductivo complejo que implica incubación durante ocho o nueve meses y retiene a sus crías sobre la boca durante la incubación, mientras que *Diplopteraster verrucosus* posee reproducción continua e incuba a sus crías en cámaras nidamentales bajo la dermis. Recolectamos individuos de *A. antarctica* del Parque Nacional Tierra del Fuego (Tierra del Fuego AeIAS, Argentina) en mayo, agosto y noviembre (etapas inicial, media y final de la incubación), mientras que los individuos de *D. verrucosus* fueron recolectados en tres campañas oceanográfica en el Área Marina Protegida de Namuncurá/ Burdwood Bank I y II. Los parámetros generales del metabolismo oxidativo (especies reactivas de oxígeno "ROS" y capacidad antioxidante total "TAC"), la densidad y el contenido energéticos se midieron en tres etapas del desarrollo de las crías. En el caso de *A. antarctica*, el consumo de energía, niveles de ROS en aumento y TAC constantes en la etapa final de desarrollo podrían actuar como una limitante al desarrollo y desencadenar la independencia de los juveniles. Del mismo modo, en los juveniles de *D. verrucosus*, que poseen nutrición materna embrionaria adicional, a pesar del continuo aumento de energía, los antioxidantes presentes en este tipo de nutrición no lograrían contrarrestar los ROS generados por el aumento del metabolismo.

Palabras clave: Embriones, estrellas de mar, metabolismo oxidativo, densidad energética, Atlántico Sur

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INTRODUCTION

Marine benthic invertebrates have diverse development modes, particularly in echinoderms, that include pelagic or non-pelagic embryos and larvae, and different modes of nutrition (particle feeding) or lecithotrophy (non-feeding) (Mileikovsky, 1971; Chia, 1974; Bosch & Pearse, 1990; Fraysse *et al.*, 2020a). Reproduction patterns and the timing and mode of nutrient provisioning to developing offspring are life-history traits that significantly affect survival and evolutionary fitness (Marshall & Morgan, 2011; Ostrovsky *et al.*, 2016).

The retention of the offspring by the adult leads to a "brooding" strategy, which is, in evolutionary terms, more derivate than the free-living larvae strategy (McEdward & Miner, 2001). Embryonic incubation on the body's surface, inside its folds, invaginations, or digestive system is known as brooding (Ostrovsky *et al.*, 2016). Brooding and viviparity are uncommon strategies and occur in a few genera of certain families, such as Asteriidae (order: Forcipulatida), Asterinidae (order: Valvatida), Ctenodiscidae (order: Paxillosida), and Pterasteridae (order: Velatida) (Lieberkind, 1926; Bosch, 1989; Bosch & Pearse, 1990; Byrne, 1996; Rivadeneira *et al.*, 2017; Fraysse *et al.*, 2018, 2020a). Due to the lack of a pelagic phase, juvenile dispersal is limited by brooding, so offspring rely exclusively on nutrition provided by the mother (McEdward & Janies, 1993). Furthermore, many studies have documented high extinction rates in brooding species (Hansen, 1983; Valentine & Jablonski, 1983; Jablonski, 1986), since these species usually have small population sizes and limited distributions, which make them primarily susceptible to environmental perturbation (Jablonski & Lutz, 1983; Liversage & Byrne, 2018).

Energy metabolism plays a critical role in an organism's survival. The allocation of energy among growth, somatic maintenance, and reproduction (Lucas, 1996) can fluctuate between sexes and during the reproductive cycle and ontogeny (Lawrence & McClintock, 1994; Pérez *et al.*, 2017; Fraysse *et al.*, 2021). The amount of energy available, its obtention, transformation, and storage rate are limited in any organism (Sokolova *et al.*, 2012). Reproductive effort in asteroids may vary according to the pattern of development. For instance, in species with a development pattern involving planktotrophy, reproductive investment is generally similar between sexes (Raymond *et al.*, 2007; Pérez *et al.*, 2010), while in species that brood, males invest energy mainly in the production of gametes, and females, in addition to the costs associated with the production of gametes, face the costs of brooding (Raymond *et al.*, 2004; Gillespie & McClintock, 2007; Pérez *et al.*, 2015; Fraysse *et al.*, 2021). Brooding represents an elevated energetic cost that involves the large, nutrient-rich eggs (Steele & Steele, 1975; Bosch & Slattery, 1999), which are presumed to be sufficient to reach the metabolic requirement for development until the embryonic stages that can feed (Shilling & Bosch, 1994; Thatje *et al.*, 2018). Although offspring incubation involves a greater energy budget, brooding benefits have been related to a higher chance of offspring survival (Vance, 1973).

The sea star *Anasterias antarctica* (Lütken, 1857) is an oral brooder species with a wide distribution, from about 40° S to the south of Isla Grande de Tierra del Fuego and the north of the Antarctic Peninsula. This species inhabits the intertidal zone up to 185 m (Bernasconi, 1964; Romanelli Michel, 2014), and frequently occurs in the community of the intertidal and subtidal zone of the coasts of the Beagle Channel (Isla Grande de Tierra del Fuego). It belongs to the highest trophic level in the Beagle Channel community associated with the kelp forests of *Macrocystis pyrifera* (Adami & Gordillo, 1999). Females of *A. antarctica* brood the lecithotrophic embryos on the oral surface over the mouth for almost nine months (Pérez *et al.*, 2015; Fraysse *et al.*, 2021) and do not feed during this period [*Anasterias minuta* (Gil & Zaixso, 2008; Gil *et al.*, 2011), junior synonym (Romanelli Michel, 2014; Mah, 2023)]. Offsprings present a connection cord that joins to a central cord that holds the metamorphic juvenile attached to the brooded mass, but there is no connection between the adult female and the brooded mass (Fraysse *et al.*, 2021).

Diplopteraster verrucosus (Sladen, 1882) is a cushion-like sea star, with uniformly reticulated supradorsal membrane and short arms. The species occurs in the South Atlantic coast (from 40° S) to Tierra del Fuego, the Burdwood Bank (BB), and the Malvinas Plateau, from 72 to 270 m depth (Clark & Downey, 1992). Females are brooders, incubating the embryos (exhibiting direct development) in a nidamental chamber (Fisher, 1940; Fraysse *et al.*, 2020b) enclosed by the supradorsal membrane and the body wall supported by skeletal elements (McEdward, 1992). Offspring probably feed from the female,

since there are projections of the brooder pyloric caeca invading the nidamental chambers, supporting a maternal extraembryonic nutrition hypothesis (Fraysse *et al.*, 2020b).

The objective was to study the energy content, energy density, and general parameters of oxidative metabolism during the development of the offspring of two brooding species with different brooding strategies: *Anasterias antarctica* (with lecithotrophy and oral incubation) and *Diplopteraster verrucosus* (with suggested maternal nutrition in nidamental chambers). We hypothesize that both species' offspring energy levels and oxidative conditions participate in the signals that trigger their release into the environment. These results can remarkably contribute to understanding maternal energy and antioxidant allocation, as well as storage utilization in the early stages of species with high maternal costs in reproduction which not only have an important trophic role but are also vulnerable to changing environmental conditions.

MATERIALS AND METHODS

Samplings of *Anasterias antarctica* (hereafter AA) brooding females (n = 74, Fig. 1A) occurred in 2017 and 2018 in May, August, and November (beginning, mid, and late incubation) from Parque Nacional Tierra del Fuego (Tierra del Fuego AeIAS, Argentina). We removed and separated the brooding mass to perform bioenergetics and oxidative metabolism measurements in each sample. Individuals of *Diplopteraster verrucosus* (hereafter DV) females (n = 43, Fig. 1B) occurred at three Oceanographic Research Cruises at Namuncurá Marine Protected Area/ Burdwood Bank and Namuncurá Marine Protected Area/ Burdwood Bank II between 2016 and 2018. Data from Fraysse *et al.* (2020b) were used, but additional individuals from subsequent campaigns were included and reanalyzed together. Twenty of the females presented nidamental chambers, where pre-metamorphic stages also occurred. We removed all post-metamorphic juveniles from the females. Since *D. verrucosus* displays continuous reproduction (Fraysse *et al.*, 2020b), we observed several cohorts within each female.

The offspring in each species were assigned to three developmental stages: (1) beginning, (2) mid, and (3) late. These stages consisted of fertilized eggs to post-metamorphic embryos in *A. antarctica* (hereafter AA 1, AA 2, and AA 3),

and three cohorts of post-metamorphic juveniles in *D. verrucosus* [hereafter DV 1, DV 2, and DV 3, following Fraysse *et al.* (2020b)].

We dried a fraction of the brooding mass of AA (n = 14, 18, and 3, for each stage respectively), and of the offspring of DV (n = 14, 31, and 13, for each stage respectively) at 60 °C to constant dry mass. After pulverization, we made pellets (0.02–0.2 g) with the dry material with a Parr 2812 pellet press (Parr Instrument Company, Moline, IL). The energy density (ED) was determined by the complete combustion of the pellets in a semi-micro bomb calorimeter (Parr, 1425), following the protocol of Lucas (1996) with modifications made by Boy *et al.* (2009). The "temperature rise" values obtained were corrected for ash and acid content and expressed as kilojoules per gram of dry mass of material free of ashes [ED, kJ g^{-1} (AFDM)]. Benzoic acid calibrations were done periodically. Energy content (EC) represents the product of ED and the wet weight.

We stored the remaining brooding masses of AA (n = 14, 14, and 12, for each stage, respectively) and offspring of DV (n = 19, 31, and 13, for each stage, respectively) at -80 °C until processing. The reactive oxygen species (ROS) and total antioxidant capacity (TAC) were determined following the method stated by Amado *et al.* (2009) with some modifications (Pérez *et al.*, 2008; Fraysse *et al.*, 2020a) by fluorometry. To quantify ROS production, we added to a 96-well microplate the reaction buffer (30 mmol L^{-1} HEPES at pH 7.2, 200 mmol L^{-1} KCl, and 1 mmol L^{-1} MgCl2) and 10 μL of the supernatant. Two wells were used per sample: in one well we added ABAP [4 mmol L^{-1} 2,2´-azobis (2-methylpropionamidine) dihydrochloride] to measure TAC, and in the other, we added ultrapure water to reach a final volume of 145 μL in each well. Then we added 10 μL of the fluorogenic probe H2DCF-DA (40 μmol L^{-1} 2´,7´ dichlorofluorescein diacetate) to each well, and fluorescence was determined at 36 °C, using a spectrofluorometer microplate reader (Fluoroskan Ascent FL 2.6, Thermo Fisher Scientific, Vantaa, Finland; equipped with Ascent software 2.6, Thermo Labsystems, Helsinki, Finland).

We performed Kruskal-Wallis tests, followed by Dunn's comparisons to analyze the variation of bioenergetic parameters (ED and EC) and the general parameters of oxidative metabolism (ROS and TAC) among three development stages for both species. Although values met the assumptions of normality and homogeneity of
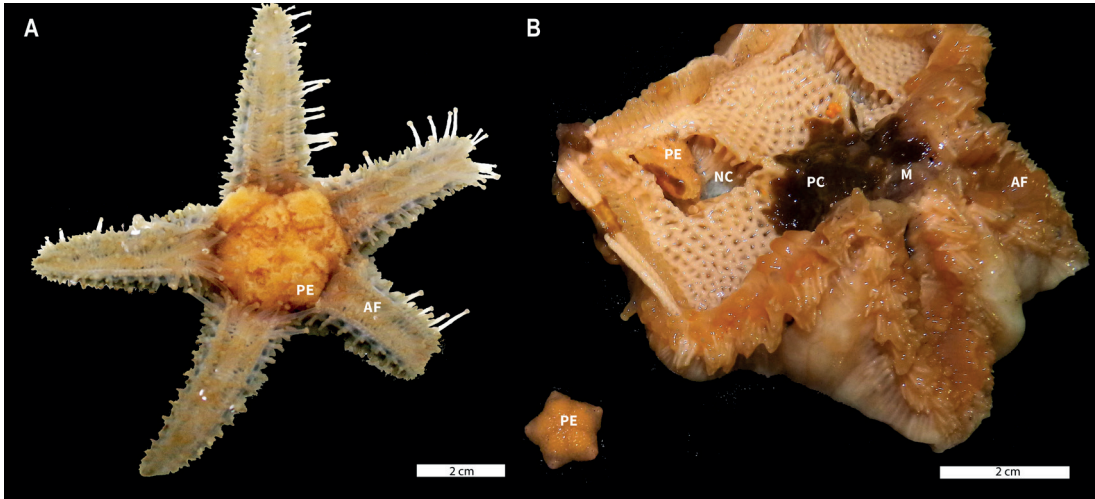
Fig. 1 (A) Oral view of a brooder *Anasterias antarctica* female, with the brooding mass on the mouth, November. (B) Oral view of a brooder *Diplopteraster verrucosus* dissection, where nidamental chambers are exposed. Abbreviations: AF, adambulacral feet; M, mouth; NC, nidamental chamber; PC, pyloric caeca; PE, post-metamorphic embryo; . Scale bars = 2 cm

variances (Kolmogorov-Smirnov and Bartlett's tests, respectively), we conducted non-parametric statistical analyses when the sample sizes were too small. Comparisons between species will be conducted qualitatively and descriptively, as stage-to-stage comparisons (*e.g.* AA 1 *vs*. DV 1) are not possible given that the defined stages are not analogous between species. We performed statistical analyses and graphs by using R statistical software, version 4.2.3 (R Core Team, 2024).

RESULTS

Energy density (ED) values were significantly different among development stages for both species, *Anasterias antarctica* and *Diplopteraster verrucosus* (Kruskal-Wallis Test, $H_{2.34}$ = 14.4, p < 0.001; $H_{2.58}$ = 12.2, p = 0.002, respectively). For the offspring of *A. antarctica*, AA 1 presented the highest values of ED, followed by AA 2, while AA 3 showed the lower value (Fig. 2; Table 1). Whereas, for *D. verrucosus* offspring, DV 3 presented the highest values, without significant differences between DV 2 and DV 1 (Fig. 2; Table 1).

Energy content (EC) values were significantly different among development stages for both species, *A. antarctica* and *D. verrucosus* (Kruskal-Wallis Test, $H_{2.34}$ = 8.99, p = 0.011; $H_{2.58}$ = 35.2, p < 0.001, respectively). For the offspring of *A. antarctica*, development stage AA 3 presented lower values of EC than AA 1 and AA 2 (Fig. 3;

Table 2). However, for *D. verrucosus* offspring, stage DV 1 presented the lowest EC value, followed by DV 2, and DV 3 showed the highest EC values (Fig. 3, Table 2).

Reactive oxygen species (ROS) were significantly different among development stages for both species, *A. antarctica* and *D. verrucosus* (Kruskal Test, $H_{2.40}$ = 18.1, p < 0.001; $H_{2.62}$ = 7.11, p = 0.029, respectively). For the offspring of *A. antarctica*, development stage AA 1 presented the lowest values of ROS, while AA 2 and AA 3 presented similar values (Fig. 4, Table 3). However, for *D. verrucosus* offspring, stage DV 1 and DV 2 presented lower ROS values than DV 3 (Fig. 4, Table 3).

Total antioxidant capacity (TAC) was not significantly different among development stages for either species, *A. antarctica* or *D. verrucosus* (Fig. 5; Kruskal Test, $H_{2.39}$ = 1.98, p = 0.371; $H_{2.63}$ = 1.16, p = 0.559, respectively).

DISCUSSION

The measurements of all analyzed variables at various stages of development indicate that these species exhibit differential energy allocation patterns and general parameters of oxidative metabolism. Here, we were able to measure energy and general parameters of metabolism as evidence of matrotrophy, which can occur through various mechanisms such as embryonic absorption, consumption of
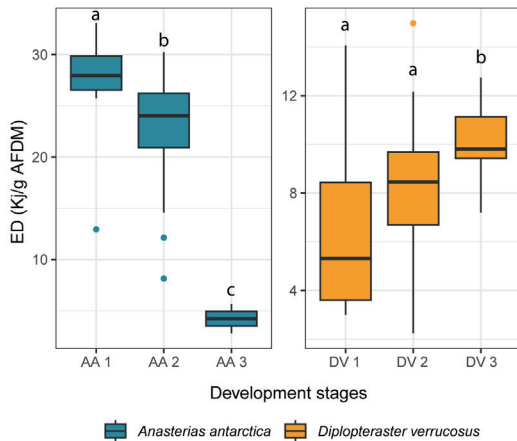
Fig. 2. Energy density (ED, kJ/g AFDM) of *Anasterias antarctica* and *Diplopteraster verrucosus*' offspring in three consecutive development stages. Median ± SE. Different letters indicate a significant difference among development stages.
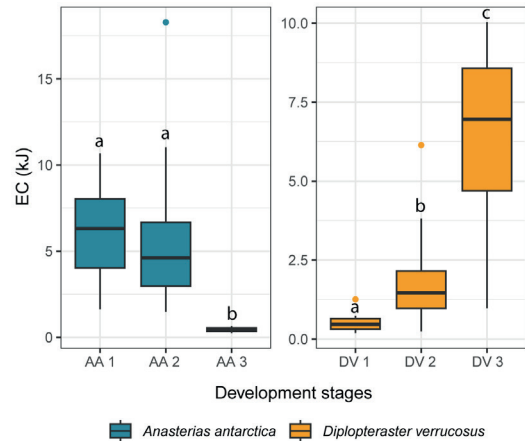

Fig. 3. Energy content (EC, kJ) of *Anasterias antarctica* and *Diplopteraster verrucosus*' offspring in three consecutive development stages. Median ± SE. Different letters indicate a significant difference among development stages.
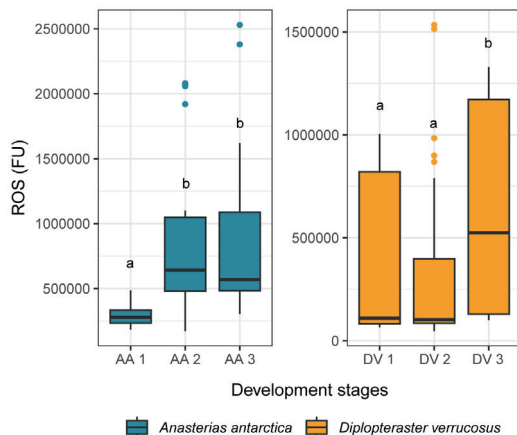

Fig. 4. Reactive oxygen species (ROS, FU = fluorescence units) of *Anasterias antarctica* and *Diplopteraster verrucosus*' offspring in three consecutive development stages. Median ± SE. Different letters indicate a significant difference among development stages.
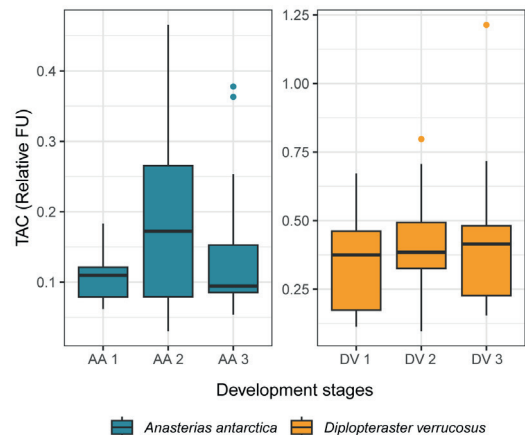

Fig. 5. Total antioxidant capacity (TAC, relative FU = fluorescence units) of *Anasterias antarctica* and *Diplopteraster verrucosus*' offspring in three consecutive development stages. Median ± SE. Different letters indicate a significant difference among development stages.

nutritional secretions, cannibalism of siblings, and digestion of maternal tissues (McClary & Mladenov, 1990; Ostrovsky *et al.*, 2016). These mechanisms ensure that the offspring receives the required nutrients for growth and development during brooding.

In the brooding mass of *Anasterias antarctica,* the energy density (ED) decreases as the incubation progresses, showing that energy reserves have depleted during the morphological, physiological, and metabolic changes that occur throughout the development process. Meanwhile, the energy content (EC) remains constant in the early stages and experiences a notable decrease towards the end of incubation. In one of the first studies on energy allocation in asteroids, using the proximal composition method, McClintock & Pearse (1986) found that the EC of embryos and the first juveniles of *Diplasterias brucei* (oral brooder species) was very similar. However, lipid content decreases, and insoluble protein content increases during development, suggesting that

Table 1. Dunn test showing Z value and p-value (in parentheses) results for energy density (ED) of the offspring development stages of the sea stars *Anasterias antarctica* (AA 1, AA 2, and AA 3) and *Diplopteraster verrucosus* (DV 1, DV 2 and DV 3). * indicates statistically significant differences.

		Development stage	
		AA 2	AA 3
Anasterias antarctica	AA 1	-2.68 (0.015*)	-3.47 (0.001*)
	AA 2	-	-2.07 (0.038*)
		DV 2	DV 3
Diplopteraster verrucosus	DV 1	-1.67 (0.094)	-3.47 (0.001*)
	DV 2	-	-2.42 (0.031*)

Table 2. Dunn test showing Z value and p-value (in parentheses) results for energy content (EC) of the offspring development stages of the sea stars *Anasterias antarctica* (AA 1, AA 2, and AA 3) and *Diplopteraster verrucosus* (DV 1, DV 2 and DV 3). * indicates statistically significant differences.

		Development stage	
		AA 2	AA 3
Anasterias antarctica	AA 1	-1.05 (0.293)	-3.01 (0.008*)
	AA 2	-	-2.45 (0.028*)
		DV 2	DV 3
Diplopteraster verrucosus	DV 1	3.32 (< 0.001*)	5.93 (< 0.001*)
	DV 2	-	2.37 (< 0.001*)

Table 3. Dunn test showing Z value and p-value (in parentheses) results for reactive oxygen species (ROS) of the offspring development stages of the sea stars *Anasterias antarctica* (AA 1, AA 2, and AA 3) and *Diplopteraster verrucosus* (DV 1, DV 2 and DV 3). *indicates statistically significant differences.

		Development stage	
		AA 2	AA 3
Anasterias antarctica	AA 1	3.64 (< 0.001*)	3.69 (< 0.001*)
	AA 2	-	0.194 (0.846)
		DV 2	DV 3
Diplopteraster verrucosus	DV 1	-0.33 (0.741)	2.15 (0.032*)
	DV 2	-	2.61 (0.009*)

protein synthesis throughout development involves lipids (McClintock & Pearse, 1986). A similar process may occur in the offspring of *A. antarctica*, where ED (energy per gram) decreases, but the EC (total energy) remains constant in the first two stages. During the third stage of development, energy consumption occurs in the brooding mass, resulting in the emergence of more visible features like spines, ambulacral feet and a fully formed digestive system (Fraysse *et al.*, 2021). These features lead to increased energy requirements for the juveniles somatic maintenance and movement within the brooding mass.

For *Diplopteraster verrucosus*, as discussed in Fraysse *et al.* (2020a), retention of different brood cohorts (stages), from pre-metamorphic to fully formed juveniles, may occur due to the occurrence of continuous reproduction along with a prolonged incubation period, as seen in *Rophiella hisrsuta* (Thatje *et al.*, 2018) and *Ctenodiscus australis* (Rivadeneira *et al.*, 2017). During the development, the juveniles ED and EC increased, particularly towards the latter stages. This increase in energy per gram (ED) and total organism energy (EC) is likely due to incorporating essential components such as lipids and proteins, which are crucial for the process of growth and development, particularly in long development processes (Pérez *et al.*, 2011). This finding indicates that the young are indeed feeding in the nidamental chambers,

which aligns with the presence of a fully developed digestive system observed in DV 3 (as well as in some individuals of DV 2, in a more advanced stage). The protrusions of the pyloric caeca of the brooding females infiltrating the nidamental chambers, as documented by Fraysse *et al.* (2020b) for this species and by McClary & Mladenov (1990) for *Pteraster militaris*, would enable maternal nutrition during these later stages of development.

Regarding oxidative metabolism, reactive oxygen species (ROS) production increases as both species develop. This increment is reasonable as the offspring become more metabolically active in later stages, leading to heightened metabolic demands during development and the consumption of antioxidants. In the case of *A. antarctica*, we observed a significant increase of ROS from AA 2 (see also Pérez *et al.*, 2015), while *D. verrucosus* manages to maintain stable ROS levels until the final stage of development (DV 3), likely related to the quality of this external provisioning from the female. Meanwhile, the total antioxidant capacity against peroxyl radicals (TAC) remains constant in both species, indicating the possibility of antioxidant synthesis or recycling in the embryo.

Furthermore, in *A. antarctica*, abnormal embryos that fail to complete development but remain attached to the brooding mass through connection cords may contribute to extra-embryonic nutrition, serving as a source of both energy and antioxidants used by the developing offspring. The contribution of the female as the source of antioxidants is not feasible in this species, as there is no observed joint between the connection cord of the brooding mass and the female. Moreover, the embryos' available energy did not increase. This connection cord that binds the brooded mass contains yolk platelets and lipid inclusions (Gil *et al.*, 2011) as was also found for the connection cord in the Antarctic sea star *Neosmilaster georgianus* by Bosch & Sattery (1999). Consumption of nutritious oocytes, deformed embryos, or cannibalism among embryos in echinoderms with internal or external brooding has been observed in *Leptosynapta clarki* (Everingham, 1961), *Pteraster militaris* (McClary & Mladenov, 1990), and *Parvulastra parvivipara* (Khan *et al.*, 2019a). Inversely, in the case of *D. verrucosus*, it is suggested that the female actively contributes to extra-embryonic nutrition through the projections of the pyloric caeca, which was also observed in *P. parvivipara* (Khan *et al.*, 2019b), thereby supporting both energy and antioxidants. It was also noted that the supply of embryonic nutrients in *P. militaris* alone was insufficient to support extended growth after metamorphosis (McClary & Mladenov, 1990). Furthermore, direct feeding from the maternal pyloric caeca by the more advanced stage may be a source of nutrition with fewer antioxidants than the initial intake in the oocyte. We hypothesize a relationship between the lower number of retained offspring from the last stage (DV 3) and the increase of ROS, in *D. verrucosus*. When this increase becomes unfavorable to the offspring, even exceeding the capacity of nutrition and protection of the female, it could induce a critical moment of development: the release of the offspring to the environment.

Moreover, both species have similar maximum sizes of mature oocytes in gonads [1.084 ± 0.028 for *A. antarctica*, and proximately 1.193 mm for *D. verrucosus* (Fraysse *et al.*, 2020b, 2021)], while the released juvenile is almost five times larger in *D. verrucosus* [proximately 1.9 mm (AA 3) for *A. antarctica* and 9.579 ± 0.370 mm (DV 3) for *D. verrucosus* (Fraysse *et al.*, 2020b, 2021)]. Matrotrophy or extra-embryonic feeding is the most plausible explanation for the energy required to support the development of these large juveniles (DV 3).

In conclusion, we suggest that as development nears completion, the juveniles approach a stress threshold due to the sustained increase in ROS production, leading to their release to the environment. In the case of *A. antarctica*, energy depletion, increasing ROS and constant TAC levels at the final development stage could act as a developmental constraint and trigger the independence of juveniles. Similarly, in *D. verrucosus* juveniles, which exhibit extra embryonic maternal nutrition, despite the continued increase in energy, the antioxidants present in this kind of nutrition do not counteract the ROS generated by the metabolic rise, also triggering the release of juveniles.

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