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Feeding and rearing techniques used for larvae of *Pleurodeles waltl* (urodele amphibian) onboard the MIR space station

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Introduction

A research group in our laboratories works on development of amphibians under microgravity conditions. Several experiments were performed in space conditions using embryos or adults of the urodele amphibian, *Pleurodeles waltl* (*Bautz et al., 1995*; *Dournon et al., 1997; Husson et al., 1998; Duprat et al., 1998; Aimar et al., 2000*). In 1999, for the so-called Perseus French space mission onboard the MIR space station, the project was to rear embryos and larvae of *Pleurodeles waltl* in microgravity conditions to study the appearance and evolution of otoconia in the inner ear *(Oukda et al., 1999a and b).* The present paper reports the technique used to feed and rear *Pleurodeles* larvae in microgravity conditions with the assistance of a cosmonaut.

Materials and Methods

Rearing of Pleurodeles in laboratory standard conditions

All the *Pleurodeles waltl* (urodele amphibian) provided from the laboratory rearing and were treated in accordance with National Legislation and The Council Directive of the European Communities on the Protection of Animals Used for Experimental and Other Scientific Purposes *(L358/1, November 24, 1980*). Embryos and larvae were staged according to the table of development of Gallien and Durocher (1957). In standard conditions, the *Pleurodeles* larvae were reared in fresh water, in plastic basins, at room temperature (18 ± 3°C) and submitted to daylight variation. The larvae were fed three times a week with nauplii of *Artemia salina, a* little crustacean arthropod, also reared and prepared in the laboratory. A few hours after each feeding, the larvae were placed in clean water.

Rearing of Artemia salina in laboratory conditions

The *Artemia salina* provided from the strain E.G. of Artemia System (Inve) defined by more than 220.000 nauplii per gram. To produce food for *Pleurodeles* larvae, about 4 g/l of *Artemia salina* cystae were placed at 25-30°C in 3 liters of water salted with 30 g/l NaCl. The water was oxygenated using air pump. Two to three days later, small nauplii hatched, swam and were attracted by light. Using a pipette, hatched nauplii were fished, filtered, rinsed and concentrated in 25 ml of soft water. They were thereafter distributed to the *Pleurodeles* larvae, which snapped up the moving nauplii. All the cystae did

not develop and nauplii could be alive in fresh water only a few hours. *Pleurodeles* larvae were routinely fed both days, and the quantity of *Artemia* was progressively adapted to the larval growing. To feed 25 *Pleurodeles* larvae reared in 3 liters of water, the mean measure was 2-3 ml of water including the concentrated nauplii.

Artemia salina preparation for the space experiment

To reduce the quantity of scraps and to facilitate hatching, the *Artemia* cystae were treated for dissolving cockles. *Artemia* were currently prepared in great quantities in the Museum-Aquarium of Nancy (France) and used to feed fish larvae and little fishes. A 7.5 kg quantity of cystae was mixed in 90 liters of fresh water added with 30 liters of a 46° chlorine solution and 495 g of sodium hydroxide pellets. Six to seven min later, the dark-brown solution became yellow and increased in temperature from 16-17 to 30°C in an exothermal reaction. When the colour was yellow, the *Artemia* were poured on a 100 µm size pore filter washed in fresh water during 10 min and placed in a 1 g/l sodium thiosulfate pentahydrate solution to stop the chlorine action. Then, the *Artemia* cystae were washed 10 min, poured in container, dried, and stocked at 4°C in a 30 % sodium chloride solution. This solution covered over the surface of the cysta preparation. When cystae were prepared recently, the percentage of hatching was about 90%.

General protocol of the space mission

The Perseus space mission occurred from February to August 1999. The most part of equipments and materials needed for our experiment, in particular a tank of physiological medium, syringes containing *Artemia* cystae with sea salt and the in live material were launched onboard a Progress automatic vessel from the Baïkonur space base. These equipments were received by the crew who had already spent one month onboard the MIR station. The in live material consisted in two batches of larvae born and developed on ground up to the neurula stage 18 and larval stage 43, and one batch of inseminated females. Onboard the MIR station, a French cosmonaut was in charge of the scientific experiments. An injection of LH-RH induced the two inseminated females to lay fertilized eggs that developed into embryos and larvae *(Aimar et al., 2000)* and constituted a third batch of larvae onboard MIR. All the *Pleurodeles* larvae of the three batches were fed with *Artemia salina* nauplii reared onboard the space station, and all the larvae were fixed during the space mission.

Conditioning of Pleurodeles larvae onboard the space station MIR.

In microgravity conditions, the embryos or larvae were continuously reared in the "egg boxes" stored in the space instrument called "Fertile" (*Gualandris-Parisot et al., 1998*). A standard egg box (height 30 mm, diameter 50 mm) was mainly composed of anodized aluminum covered with Teflon. A Delrin grid separated the internal volume in two chambers, an air one (18 ml) and a water/egg one (15 ml) closed by a polycarbonate transparent cover (Fig. 1). In the middle of the grid, an orifice with a detachable plug could receive the extremity of a syringe used to introduce or aspirate part of water. The standard egg boxes were designed to put 8 ml of sterile water and 20 eggs or young embryos with their jelly coat into the water-egg chamber.

In this flight experiment, the number of larvae placed into the egg boxes depended on the size of the larvae at the beginning and at the end of the experiment. When the initial stages were fertilized eggs and neural stage 18, there were 10 maximum per box. When the initial stage was the larval stage 43, an additional cylinder (17 ml) increased the air volume of the egg boxes that contained 3 larvae (Fig. 1). All the *Pleurodeles* embryos and larvae were continuously reared at 18°C in the sterile rearing Steinberg medium (*Steinberg, 1957*), and in darkness except during the phases of feeding and medium change.

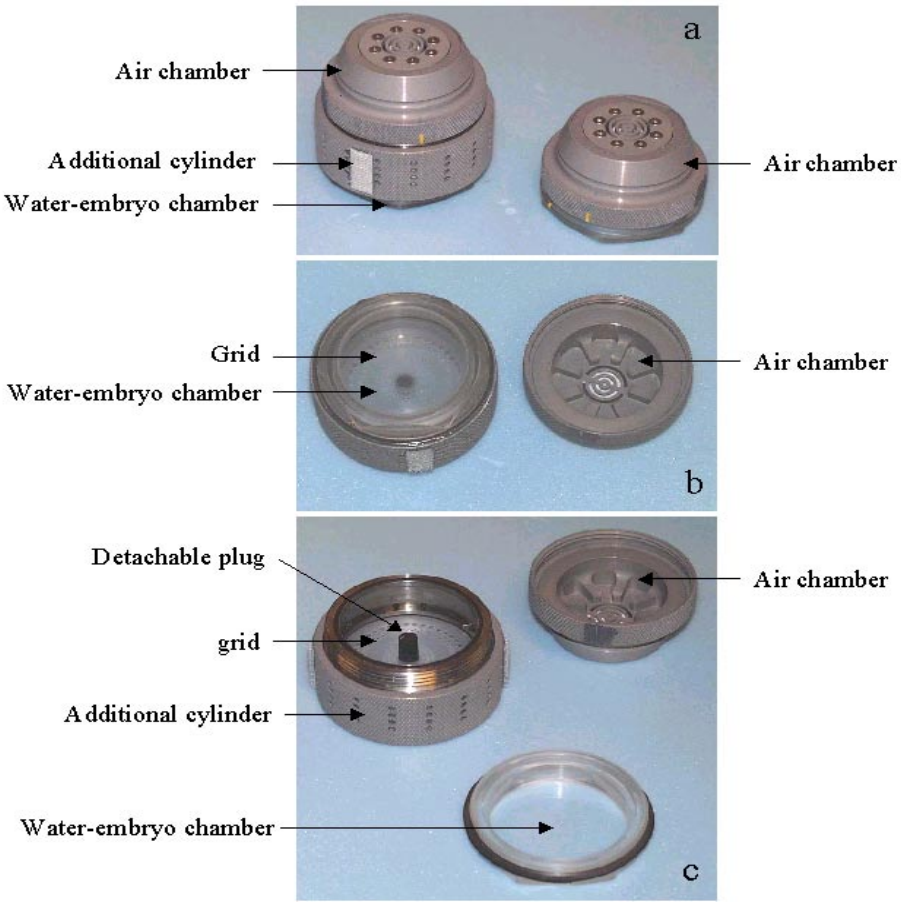


Fig. 1. a. left: egg box with an additional cylinder; right: standard egg box.
b: standard egg box, water chamber closed and air chamber open.
c: egg box with an additional cylinder, air chamber open and enlarged with the cylinder; water chamber open.

Results

Number of Artemia nauplii required to feed Pleurodeles larvae

In the ground laboratory, the mean number of *Artemia* needed to feed one *Pleurodeles* larva was experimentally defined in function of larval stages of development. The results were used to define the total number of hatched nauplii in function of the number of *Pleurodeles* larvae per egg box (Table 1).

Larval stage	Mean number of nauplii eaten per larva	Mean number of nauplii eaten by 10 larvae	Mean number of nauplii eaten by 3 larvae	Distributed volume of medium including nauplii	Number of needed syringes
37-38	5 ± 1	50 ± 10		0.5 ml	0.5
39-40	10 ± 2	100 ± 20		1 ml	1
41-42-43	15 ± 3	150 ± 30		1 ml	1
44	20 ± 4	200 ± 40		1.5 ml	1.5
45	20 ± 4		60 ± 12	0.5 ml	0.5
46-47	30 ± 5		90 ± 15	0.75 ml	1
48-49	40 ± 7		120 ± 21	1 ml	1

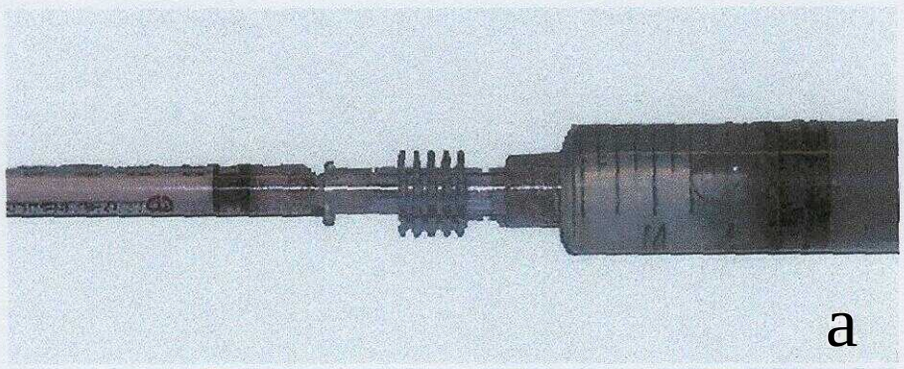
Table 1. Mean number of *Artemia salina* nauplii needed to feed *Pleurodeles waltl* larvae depending on the larval stages of development, and volume of medium including nauplii distributed per box. The feeding occurred every two days.

Method used to rear Artemia onboard the MIR space station

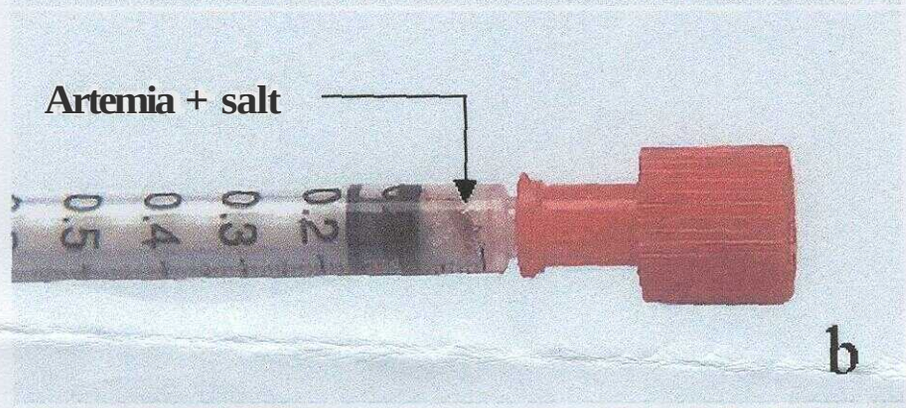
To obtain hatching of *Artemia* nauplii in hermetic containers, different forms of container with various proportions of air/liquid phases were tested. The optimal result was obtained with long and cylindrical tubes**.** For ergonomical reasons, we selected three kinds of syringes equipped with a Luer-Lock system. The advantage of this system was the possibility of connection between two syringes (Fig. 2a). One ml syringes were used to store both *Artemia* cystae and sea salt before rearing, 10 ml syringes were used to rear *Artemia*, and 8 ml syringes were used to pump out of a tank a sterile physiological medium, the rearing Steinberg medium *(Steinberg, 1957)* and to put it in the other syringes.

Number of Artemia nauplii per syringe

When *Artemia* cystae and sea salt crystals were stocked without water, the cystae could be conserved up to three months at the 25-27°C MIR ambient temperature. However, as the mean number of hatching *Artemia* nauplii decreased depending on the storage duration, the quantity of *Artemia* cystae per syringe was corrected. Five mg of *Artemia* cystae and 150 mg of sea salt crystals were put inside each 1 ml syringes (Fig. 2b), then cystae and salt were diluted in 5 ml of physiological medium in 10 ml syringes, and 5 ml of air were also added. The mean number of hatched nauplii was estimated to 300 nauplii in 5 ml of physiological medium after two months of storage in 1 ml syringes at MIR ambient temperature (Fig. 2c).



a



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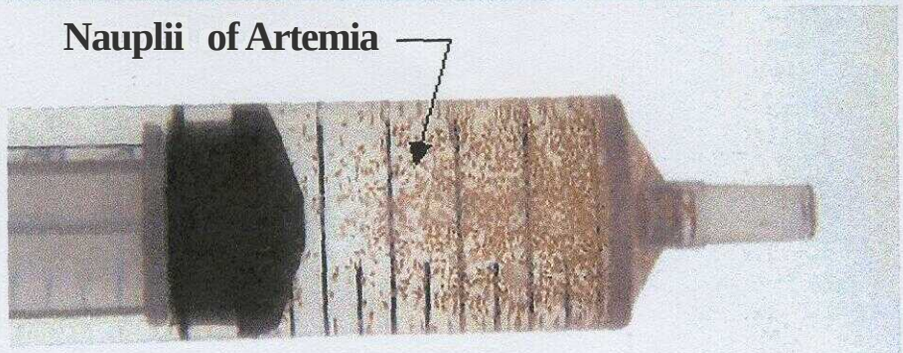


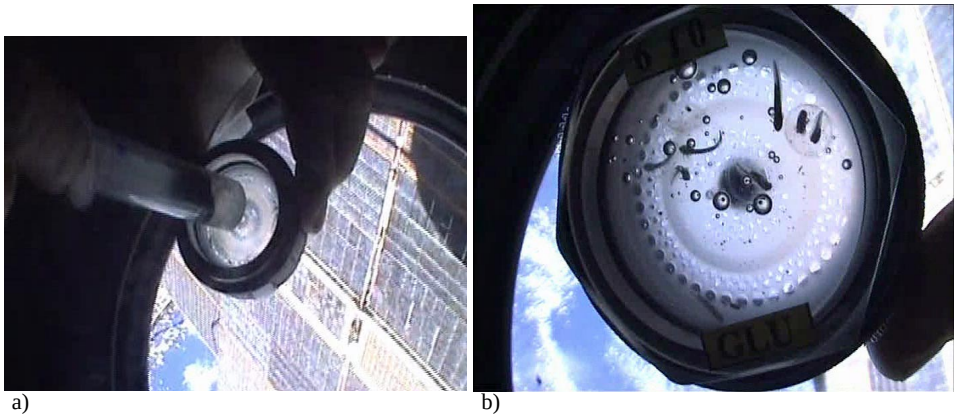
c

Fig. 2. a: Luer-Lock system connecting 1 ml and 10 ml syringes.
b: detail *of Artemia salina* cystae and sea salt contents stocked in a 1 ml syringe.
c: detail of a 10 ml syringe containing in live *Artemia salina* nauplii.

A protocol of management of syringes and distribution of food was built, tested on ground and used during the space mission. For the in flight experiment and synchronous ground control, a total of 70 syringes containing *Artemia* cystae and salt was prepared and stored on ground at 8°C during four weeks and then in flight at the MIR ambient temperature during two months.

In flight Artemia rearing and food distribution

A 8 ml syringe was refilled by the cosmonaut at the tank with a physiological medium and was used to temporarily stock the medium in a glove box. This syringe was connected to a 10 ml syringe in which 5 ml of physiological medium were injected. Then, the 10 ml syringe was connected to a 1 ml syringe containing *Artemia* cystae and salt. When the 10 ml and 1 ml syringes were connected, the inside of the 1 ml syringe was washed three times using the two pistons in alternation, and finally the 5 ml of now salted medium was stored with the *Artemia* cystae in the 10 ml syringes. Five ml of air were added in the 10 ml syringe by pulling the piston. When the 10 ml syringes were prepared, they were placed at MIR ambient temperature during three days. The medium induced the cysta development, but some cystae (about 10%) did not develop. Three days later, before food distribution, a 41 µm size pore filter (Ref NY4104700, Millipore S.A., Molsheim, France) was connected to the 10 ml syringe including in live *Artemia* nauplii. The salt medium was eliminated and the nauplii were kept on the filter. The 10 ml syringe was replaced by a 1 ml dispenser and the 8 ml syringe containing sterile physiological medium was connected at the other side of the filter. Before feeding *Pleurodeles* larvae, nauplii were collected transferring 1 ml of physiological medium from the 8 ml syringe into the 1 ml dispenser through the filter. In a third time, the cosmonaut opened the egg box, air chamber side, and put on the grid plug. Hereafter, he connected the dispenser containing the in live nauplii to the middle orifice of the egg box grid and put medium and nauplii to feed the *Pleurodeles* larvae (Fig. 3a and b).



a) b)

Fig. 3. a: cosmonaut cleaning an egg box.
b: egg box containing in live larvae seen by transparency in front of a porthole of the MIR space station.

In standard rearing conditions, the swimming larvae eat nauplii for the first time at stages 37-38. In flight, the larvae were fed from the estimated day for stage 37 at 18°C. The quantity of nauplii and consequently the quantity of medium including nauplii distributed per egg boxes was adapted for space conditions. After the washing of nauplii, the loss of nauplii was estimated at 50%. Consequently, 150 nauplii were considered alive in the 1 ml dispenser and put in the egg boxes (Table 1).

Renewal of clean physiological medium

In standard ground rearing as in egg boxes, the *Pleurodeles* larvae did not eat all the nauplii. Consequently, on ground, it was necessary to renew all the water after each feeding. In flight, before the feeding occurring every two days, the medium of each box was filtered through a 0.22 µm size pore filter (Millex-GS, Millipore, Molsheim, France) and reinjected by a pump operated by the cosmonaut. During this operation, a part of dead nauplii and non-developed cystae was eliminated, and about 2 ml of medium were renewed. Nauplii were then distributed to larvae (Fig. 3a).

Pleurodeles larvae obtained after the space flight

The experiment protocol stipulated that the cosmonaut fixed the surviving embryos or larvae when the number of animals was reduced by half. In ground laboratory, the same protocol was applied. In flight, 79 of 117 (67.5%) animals, and on ground, 71 of 109 (65.1%) were fixed for further analysis. Among these animals, 39 on 117 (33.3 %) and 21 of 109 (19.2%) were fed 1 to 8 days and 1 to 12 days, respectively (Table 2).

Discussion

The general constraints required for space flights are a minimum of weight and volume for materials. Concerning ergonomy, materials must be simple to use by experimenter cosmonauts, which performed the operations with a minimum of actions and in all safety. These requirements limit the scientific possibilities.

For this experiment, the number of developed embryos and larvae was comparable onboard the MIR space station and the ground laboratory. However, we expected larvae at later developmental stages. The difficulties of older larvae obtaining derived from technical problems of rearing. In the egg boxes, all the dead nauplii and no-hatched cystae were not extracted by the aspiration process. As the renewal of a part of the physiological medium occurred every two days, the scraps could induce the death of one or more *Pleurodeles* larvae provoking the death of other animals in the same box.

It was the first time that larvae of urodele amphibians were reared and fed in microgravity conditions. In the anuran amphibian *Xenopus laevis*, tadpoles were previously reared and fed in microgravity *(Sebastian et al., 1998*). For carnivorous *Pleurodeles* larvae, in live food must be prepared and periodically distributed, whereas for phytophagous *Xenopus* tadpoles food such *Euglena* algae could be included in mini-aquariums for all a space flight *(Sebastian et al., 1998)*. For *Pleurodeles*, the rearing medium must be changed at each food distribution but not for *Xenopus*. Consequently, this technique of *Artemia* rearing could be used, but should be adapted and improved in further experiments concerning urodele larvae, as *Pleurodeles waltl* or *Cynops phyrrogaster* when used in Japanese and American space experiments (*Wiederhold et al., 1995).*

Summary

In laboratory standard conditions, *Pleurodeles waltl* larvae (urodele amphibian) were reared in basins with clean fresh water at room temperature and submitted to daylight variation. They were periodically fed with nauplii of *Artemia salina* developed from cystae in salted water. In space flight conditions, the *Pleurodeles* larvae were continuously reared at 18°C in hermetic boxes and in darkness except during the phases of feeding and medium changing. To produce onboard the MIR space station in live food in adequate quantity for the larval *Pleurodeles*

	Animals submitted to microgravity from:			Fixation of animals in microgravity at:			Duration of in flight development (days)	Duration of in flight feeding (days)
	Stage	Age (days)	Number	Stage	Age (days)	Number (% of surviving)		
On board	Eggs	0	10	32-33	10	10 (100%)	10	0
	Eggs	0	10	34	13	2 (20%)	13	0
	Eggs	0	10	36	16	4 (40%)	16	0
	Eggs	0	10	37	19	4 (40%)	19	1
	Eggs	0	10	38	20-21	10 (100%)	20-21	2-3
	18	3	12	32	8	12 (100%)	5	0
	18	3	10	32-33	10	2 (20%)	7	0
	18	3	10	33-34	13	5 (50%)	10	0
	18	3	10	34	18	5 (50%)	15	0
	18	3	10	39	22	10 (100%)	19	4
	43	33	9	45	38	9 (100%)	5	5
	43	33	6	45	41	6 (100%)	8	8
Control on ground	Eggs	0	10	32-33	10	10 (100%)	10	0
	Eggs	0	10	34	13	10 (100%)	13	0
	Eggs	0	10	36	16	10 (100%)	16	0
	Eggs	0	10	37	19	3 (30%)	19	1
	Eggs	0	10	38	21	3 (30%)	21	3
	18	3	10	32-33	10	10 (100%)	7	0
	18	3	10	36	16	10 (100%)	13	0
	18	3	10	39	22	2 (20%)	19	4
	18	3	10	41	27	3 (30%)	24	9
	18	3	10	42	30	1 (10%)	27	12
	43	33	6	44	37	6 (100%)	4	4
	43	33	3	45	41	3 (100%)	8	8

Table 2. Duration of the in flight development and feeding depending on the initial stages of development at the beginning of the experiment.

growing, the mean number of *Artemia* nauplii was firstly defined on ground depending on larval stages of *Pleurodeles* development. Then, the optimal conditions of rearing in hermetic boxes were tested. *Artemia salina* cystae and sea salt were stocked without water in syringes up to three months. Cystae were activated with a physiological medium three days before use. The protocol of management of the syringes and distribution of food performed by a cosmonaut is described.

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