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RESEARCH ARTICLE

N. Fockedey · A. Ghekiere · S. Bruwiere · C. R Janssen
M. Vincx

Effect of salinity and temperature on the intra-marsupial development of the brackish water mysid *Neomysis integer* (Crustacea: Mysidacea)

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Abstract The brackish water mysid *Neomysis integer* has been proposed as a toxicological test species for the low saline reaches of Western European estuaries and brackish inland water bodies. The embryonic/larval development is a critical time window within the life history of an organism and has high potential to serve as a tool for assessing endocrine-disruptive effects. A protocol is developed to examine the intra-marsupial development of *N. integer* in vitro, and a morphological description of the embryonic and larval developmental stages was made. Daily survival percentage, percentage survival days, hatching success, total development time, duration of each developmental stage and the size increment of the embryos and larvae were evaluated as potential endpoints, and their response to temperature and salinity was investigated. The survival and hatching success are highly dependent on the salinity conditions, while the development time is strongly affected by temperature. High temperatures (21°C) shorten the development time in comparison with low temperatures (11°C) from 22 to 10 days, but have an opposite effect on survival. Optimal salinity for in vitro embryonic/larval development of *N. integer* is 14–17. Living in lower or higher salinities thus implies suboptimal conditions for the juvenile recruitment to the population, unless the species can actively regulate the concentration of its marsupial fluid. The developed in vitro technique may be used for testing the effect of both abiotic factors and (endocrine) disrupting chemicals on the intra-marsupial development of *N. integer*. Survival, hatching success and development time appeared to be adequate endpoints, while size and growth increment of the embryos/larvae seemed to be unsuitable.

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N. Fockedey · S. Bruwiere · M. Vincx
Biology Department, Marine Biology Section, Ghent University, Krijgslaan 281/S8, 9000 Ghent, Belgium

A. Ghekiere · C. R Janssen
Laboratory of Environmental Toxicology and Aquatic Ecology, Ghent University, J. Plateaustraat 22, 9000 Ghent, Belgium

N. Fockedey (✉)
Flanders Marine Institute (VLIZ), Wandelaarkaai 7, 8400 Oostende, Belgium
E-mail: Nancy.Fockedey@vliz.be

Introduction

In the last decade, concerns have been expressed about the potential effects of low levels of (natural and anthropogenic) endocrine-disrupting chemicals on man and the environment. The effects on wildlife and especially on invertebrate species might have far-reaching adverse consequences for biodiversity and the sustainability of natural ecosystems (e.g. Guillette and Guillette 1996; Edmunds et al. 2000; Santos et al. 2002). Industry and environmental management agencies urgently need new tools to assess the potential of effluents and chemicals to perturb the hormonal system (Depledge and Billinghurst 1999). Because of the high contamination loads in estuaries, the need to use estuarine organisms in ecotoxicological studies is stressed (Lawrence and Poulter 2001). Through their adaptation to the dynamic estuarine environment, these animals may either be preadapted to tolerate pollution stress or be more susceptible to any additional stress (Lawrence and Poulter 1996). The brackish water mysid *Neomysis integer* has been put forward as a test organism for the evaluation of environmental endocrine disruption in the brackish reaches of Western European estuaries and inland water bodies (Verslycke et al. 2004). Since *N. integer* has a key function in the estuarine ecosystem (Mees et al. 1994; Hostens and Mees 1999; Maes et al. 2003), an alteration in the intra-marsupial development due to changing abiotic environmental conditions or pollution might have an impact at the population level on the species' recruitment and thus on the sustainability of the estuarine ecosystem (e.g. Depledge and Billinghurst 1999; Lawrence and Poulter 2001).

As reproduction and embryonic/larval development are critical time windows within the life history of an organism (Depledge and Billinghurst 1999; Lawrence and Poulter 2001), they have high potential to serve as sensitive indicators of endocrine disruption (Wittmann 1984). They are the critical stages in the hierarchical levels of response by an organism to pollution that link molecular, subcellular and physiological responses to population and community impact. The number of endocrine-disrupting chemicals identified to specifically affect the development, fecundity and reproductive output of aquatic invertebrates is increasing (a.o. Sundelin and Eriksson 1998; Lawrence and Poulter 2001; Billinghurst et al. 2001; Kast-Hutcheson et al. 2001; Nice et al. 2003; Roepke et al. 2005; Forget-Leray et al. 2005).

Neomysis integer, like all other mysid species, carries its embryos in a marsupium where the entire larval development from oviposition to the release of free-swimming juveniles takes place (Wittmann 1984). It allows the embryos/larvae some degree of protection against predation. The marsupium is a chamber formed by thin-walled concave plates fringed with long setae that interlock ventrally to form a closed chamber (Mauchline 1980). Studying the intra-marsupial development in vivo, i.e. through the semi-transparent oostegites, is difficult (N. Fockedey, personal observation) or requires anaesthetization of the test specimens (Irvine et al. 1995). These difficulties emphasize the need for the development of a protocol to study intra-marsupial development in vitro. Although some in vitro data are available on the development time and the hatching success of *N. integer* embryos at a salinity range of 0.4–16 at 15°C (Vlasblom and Elgershuizen 1977), detailed information is lacking for a wider salinity range in combination with a wide temperature range. *N. integer* is known to be euryhaline, tolerating salinities of 1–40 (Vlasblom and Elgershuizen 1977), and eurythermic tolerating temperatures between 0 and 30°C under laboratory conditions (Arndt and Jansen 1986; Mauchline 1980). The combined influence of temperature and salinity on the intra-marsupial development as well as their optimal range have to be known, in order to develop optimal laboratory cultures and to differentiate between chemically induced variability and natural variability in toxicity testing. The in vitro embryogenesis and larval development have been described in other mysids and pericaridans, and the technique is used as a bioassay to evaluate changing environmental conditions (Vlasblom and Bolier 1971; Morritt and Spicer 1996a), toxicity and endocrine disruption (Lawrence and Poulter 2001).

The aim of this study is to develop and optimize a methodology to study the intra-marsupial development of *N. integer* in vitro. A detailed description of the embryonic mortality, morphology and the duration of subsequent developmental stages are obtained. The combined impact of salinity and temperature on the intra-marsupial development is studied on endpoints like survival, hatching success, duration of development and size of the embryos and larval substages. These results are essential for the development of a bioassay to assess the effects of endocrine-disrupting chemicals on the intra-marsupial development of *N. integer*.

Materials and methods

Sampling

The brackish water mysid *N. integer* used in the experiments originates from dock B3 in the harbour of Antwerp (Belgium), situated at the right bank of the Schelde river and connected to it through the Berendrecht and Zandvliet sluices. A chemical factory pumps up water from the dock for use as cooling water. Animals and debris are extracted from this incoming water by sieving over a 1×1 mm^2 sieve and collected in a reservoir. Living *N. integer* were collected with a hand net (2×2 mm^2 mesh size) from this reservoir and transported to the laboratory within 2 h. Salinity and temperature conditions in the reservoir during the sampling period (weekly from 16 March to 20 April 2004) were on average 5 psu and 11°C. In the laboratory the animals were kept in a 16°C climatized room for <7 days at a density of ± 50 ind l^{-1}. They were fed *ad libitum* on <24 h old *Artemia* nauplii and the culture water was replaced every 2–3 days (5 psu artificial seawater—Instant Ocean®, Aquarium Systems, France).

Description of the intra-marsupial development

Detailed descriptions of the histology of successive larval stages of *N. integer* are given by Wagner (1896) and Needham (1937). External morphological descriptions of the intra-marsupial development are available in Kinne (1955) and de Kruif (1977). Although these papers give the morphological descriptions, they do not contain any pictures or drawings which could be useful tools for ecotoxicological evaluation. Gravid females with embryos/larvae at different stages were selected from the field samples. Using a stereomicroscope, the animals were decapitated and the embryos/larvae were removed from between the lamellae of the marsupium with a fine spatula while submerged. The embryos/larvae were then individually transferred using a Pasteur pipette to a Petri dish (diameter 38 mm, height 4 mm) containing 4 ml of aerated artificial seawater (salinity 5) at 16°C. Preliminary experiments indicated a significant higher survival of the embryos when placed on an orbital shaking table (80 rpm); this approach was used for all subsequent testing. Daily, half of the water was renewed and photographs were taken to aid in the description of the intra-marsupial development.

Short-term survival

To select the adequate temperature and salinity range for the experimental design, a multifactorial experiment was

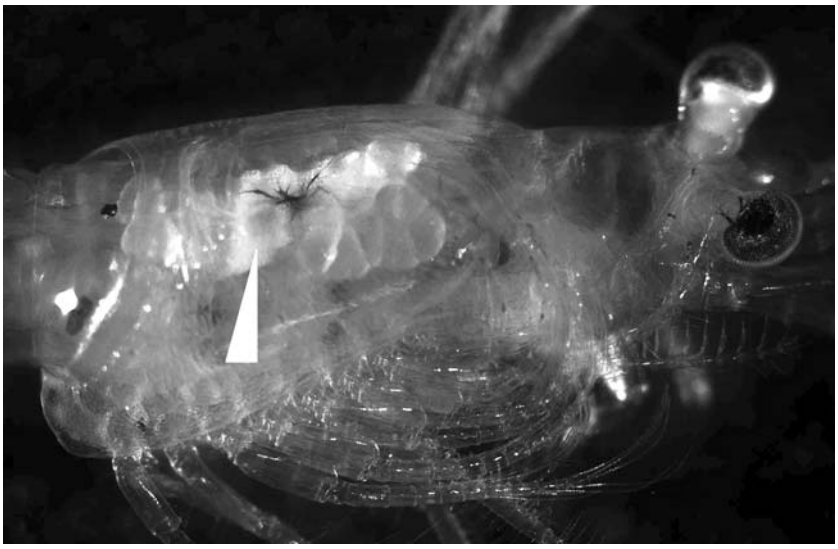

Fig. 1 The ripe ovary fills the posterior dorsal lateral regions of the thorax (*white arrow*) and can easily be observed under a stereomicroscope (12×) as a *white mass* with clear egg contours

performed to evaluate the embryo's survival during a 3 day period. Spherical shaped (stage I) embryos of 24 field-collected gravid females were taken from the brood pouches (between 18 and 105 embryos per brood) and randomly distributed to Petri dishes (25 embryos per dish) containing 4 ml aerated artificial seawater. Embryo survival was monitored during 3 days. The following test-design was used: 13, 16 and 19°C; each of these temperatures tested at salinity 2.5, 5, 10, 15, 15, 20 and 25 (two replicates per treatment). Petri dishes were placed on an orbital shaking table (80 rpm). No prior acclimation to the experimental salinity or temperature was done. Half of the culture medium was renewed daily and dead (i.e. white and/or shrivelled) embryos removed. After 72 h, survival was noted and expressed as the mean percentage survival.

Mating and fertilization

Non-gravid females, with a standard length (from the tip of the rostrum in between the eye stalks to the end of the last abdominal segment) of 11–15 mm and with a large ovary, were selected from the field samples. The mature ovary fills the posterior dorsal lateral regions of the thorax (Mauchline 1980; Fig. 1). The whitish eggs present in the ovary can easily be observed through the carapace. Mature males (standard length 11–13 mm) were distinguished by their elongated fourth pleopods that are stretched to the end of the last abdominal segment and the paired penes.

To allow fertilization, one female was placed with two males in a 400 ml glass container filled with 350 ml artificial seawater (salinity 5). The jars were placed in a climatized room at 16°C under a 12 h light:12 h dark light regime. The following was performed daily: excess food, faeces and possible moults were removed, dead mysids taken away and replaced by new individuals; 80% of the medium was renewed and fresh food was added (1,000 *Artemia* nauplii mysid^{-1}).

Mating takes place at night (Mauchline 1980) and coincides with the moulting of the female (Wittmann 1984). Females with full marsupia were isolated for 2 days prior to removal of the embryos from the marsupium on day 3.

Table 1 Percentage survival days (±standard error), median percentage survival, mean percentage hatching (±standard error) and percentage of replica leading to hatching for all treatments of the Central Composite Design

Treatment	Block	Temperature (°C)	Salinity	Percentage survival days (% day^{-1})	Median percentage survival (days)	Hatching success (%)	Replica hatched (%)
A	1	16.0	15.0	55.6 ± 7.7	7.3	52.2 ± 9.1	83.3
F	1	16.0	15.0	29.3 ± 7.5	5.1	37.5 ± 14.5	41.7
I	2	16.0	15.0	64.5 ± 5.5	14.6	53.9 ± 4.6	91.7
J	2	16.0	15.0	71.7 ± 5.0	16.0	48.4 ± 4.6	100.0
Average control		16.0	15.0	55.3 ± 3.9	15.6	49.5 ± 3.6	79.2
B	1	13.0	8.0	24.1 ± 3.3	5.8	25.8 ± 7.6	16.7
C	1	19.0	22.0	28.1 ± 4.2	4.7	16.0 ± 4.3	28.6
D	1	13.0	22.0	38.3 ± 9.5	5.9	49.9 ± 12.0	46.2
E	1	19.0	8.0	21.2 ± 2.2	4.5	20.0 ± 0.0	6.3
G	2	16.0	5.1	40.9 ± 1.8	4.2	0	0
H	2	16.0	24.9	33.8 ± 2.8	6.4	0	0
K	2	11.7	15.0	71.6 ± 6.1	25.4	57.4 ± 6.2	100.0
L	2	20.2	15.0	66.6 ± 3.9	10.3	36.4 ± 4.5	100.0

Table 2 Morphological features and corresponding activity of the intra-marsupial stages of *Neomysis integer*

Morphology	Yolk	Activity	Present study, Kinne (1955) and Mauchline (1973)	De Kruif (1977)	Wittmann (1981)
Egg-like, sub-spherical (Fig. 2a); first 2 days in two packages within a tertiary egg membrane Later: cephalic and abdominal rudiments developing (Fig. 2b)	Yolk granules spread all over embryo	Inactive	stage I = 'egg'	stage I	E1–half E5
Shedding of egg membrane Comma-shaped habitus with rudimentary pointed abdomen clearly distinguished from rounded anterior; appearance of two pair of rudimentary thoracic appendages and abdominal setae (Fig. 2c) Later: the beginning of abdominal segmentation, without appendages (Fig. 2d)	Yolk granules homogeneously spread all over embryo	Inactive	stage II = naupliar stage = 'eyeless' larva	stage II stage III stage IV	Half E5 + N1 to N4
Further extension of the body and elongation thoracic appendages (Fig. 2e); appearance of cleft at optic rudiment (Fig. 2f)	Yolk migrates dorsally	Inactive			
Development of head; optic lobes with pigmented eye rudiments; rudiments of telson and uropods visible; further segmentation of abdomen; brown chromatophores appearing laterally (Fig. 2g, h)	Yolk diminishes and migrates dorsally in the anterior part	Rhythmic contractions of the gut and beating of the heart		stage V	
Moulting from naupliar cuticle Distinct eye projections; development of uropods and pleopods; developing eight thoracic appendages, mouthparts and antennae; developing carapace and elongated abdomen (Fig. 2i, j)	Yolk disappears	Very active flexing and stretching of the body; moving of the appendages	stage III = post-naupliar stage = 'eyed' larva	stage VI	P1–P3
Moult All (except sexual) characteristics similar to adult (Fig. 2k)	No yolk left; actively feeding	Freely swimming	Juvenile	Juvenile	Juvenile

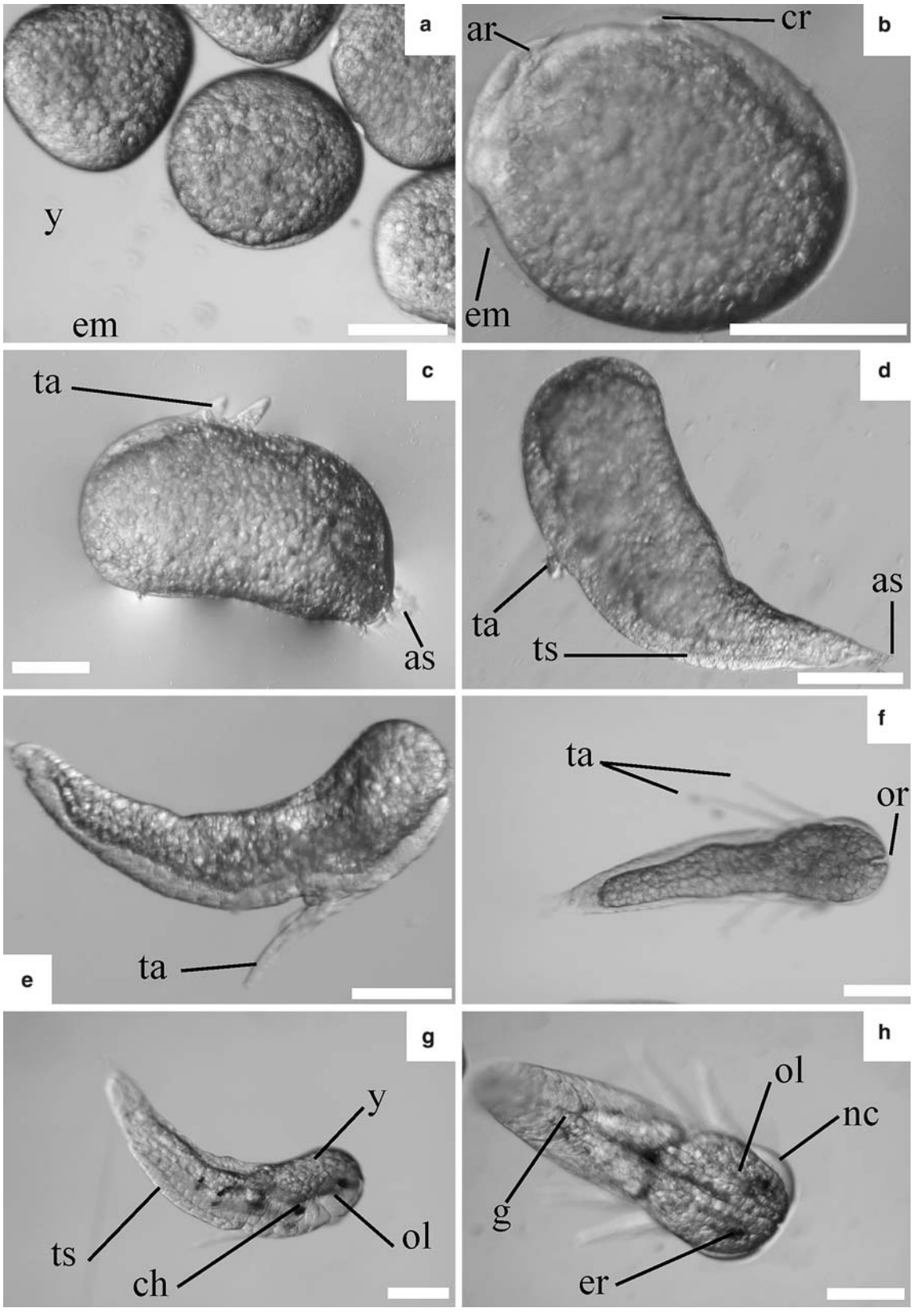

Fig. 2 Embryonic stages of *Neomysis integer*: stage I (**a**, **b**), stage II (**c**–**h**), stage III (**i**, **j**) and the free-living juvenile (**k**). (*an* antennae; *ar* abdominal rudiment; *as* abdominal setae; *car* carapace; *cr* cephalic rudiment; *ch* thoracic chromatophore; *er* eye rudiment; *em* egg membrane; *g* gut; *m* mouth parts; *nc* naupliar cuticle; *or* optic rudiment; *ol* optic lobe; *pl* pleopods; *t* telson; *ta* thoracic appendages; *ts* thoracic segmentation; *u* uropods; *y* yolk granules). *Scale bar* = 250 μm

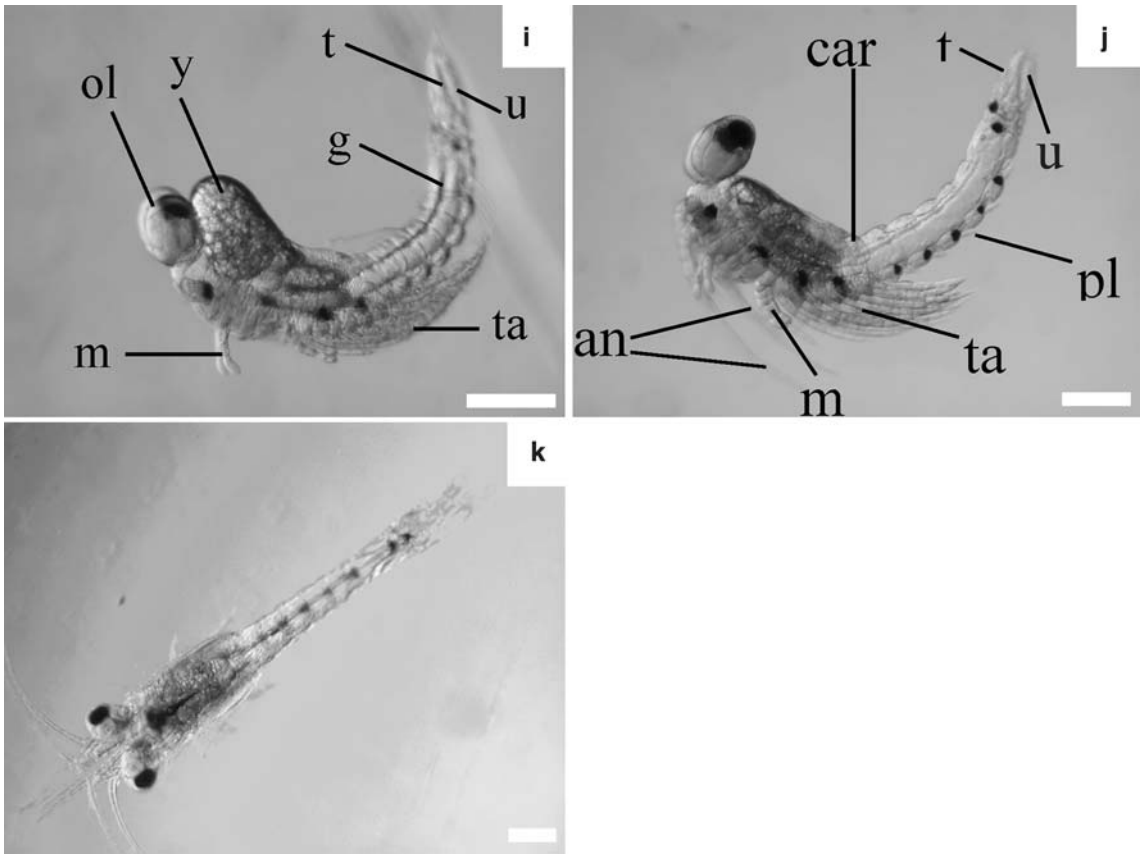

Fig. 2 (Contd.)

Effect of salinity and temperature on intra-marsupial development

The evaluation of the effects of salinity and temperature on the in vitro intra-marsupial development of *N. integer* was done using a (circumscribed) 'Central Composite Design' (STATISTICA 6.0). For the two independent factors—i.e. salinity and temperature—12 treatments were tested in two blocks of 6 with each block comprising two central points (Table 1). Subsequently a response surface model was fitted to the data and the optimal temperature/salinity combination for the different endpoints tested was derived.

To achieve randomization the 3-day-old embryos of one brood were distributed in as many treatments as possible and transferred to the 5 ml wells of a 12-cell multiwell plate. All treatments were performed in 12 replicates; each replicate containing 7 to 17 embryos. No adaptation period to the experimental salinity or temperature was done prior to the test, but animals surviving < 3 days were excluded from the analyses. Multiwell plates were placed on an orbital shaking table

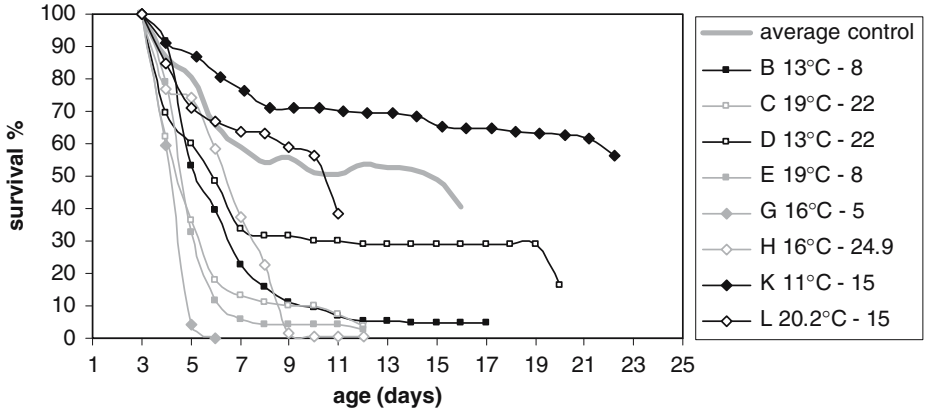

Fig. 3 Survival functions of all treatments (temperature°C–salinity). In *grey*: mean of the centre point treatments

Table 3 Mean survival percentage [± standard error (SE)] of stage I embryos after 72 h

Temperature (°C)	Salinity					
	2.5	5.0	10.0	15.0	20.0	25.0
13.0	10 ± 6	20 ± 4	32 ± 4	60 ± 8	58 ± 2	48 ± 0
16.0	8 ± 0	16 ± 8	46 ± 10	64 ± 4	40 ± 0	28 ± 4
19.0	2 ± 2	8 ± 8	26 ± 10	28 ± 4	26 ± 6	20 ± 12

(80 rpm) and covered from the light. The following was performed daily: the survival and developmental stage were noted, dead embryos/larvae were removed, half of the medium was replaced with freshly prepared medium and the salinity and temperature was monitored. Every day (maximally eight) embryos/larvae were measured per replicate using a drawing mirror mounted on a stereomicroscope (25–50×). The following measurements were performed with ImageJ 1.32e (http://rbs.info.nih.gov/ij/Java 1.3.1_03 public domain): the maximum diameter (i.e. Feret's diameter) of stage I embryos, the total length (TL) excluding the abdominal setae in stage II larvae and the total length (from the eye basis to the tip of the uropods) in stage III larvae. Hatched juveniles were fixed in 4% formaldehyde and their standard length (SL), from the eye basis till the last abdominal segment, measured dorsally.

Statistics

The cumulative survival function was plotted as percentage survival. The percentage survival days (Jones 1972) and the median survival time (50% survival) were calculated for each treatment. Survival percentage data of the four control treatments were submitted to a two-factor ANOVA (treatment × age of embryo/larvae) without meeting the normality assumptions (Mann and Harding 2003). Other endpoints, like percentage survival days, hatching success and development time, were tested between treatments using a one-factor ANOVA. Fisher's multiple comparison test was used for post-hoc comparison when appropriate. The size of the embryos, larvae and juveniles, and their relative growth (in % day^{-1}) was tested for each substage using a two-way ANOVA taking age into account. All replicate values of

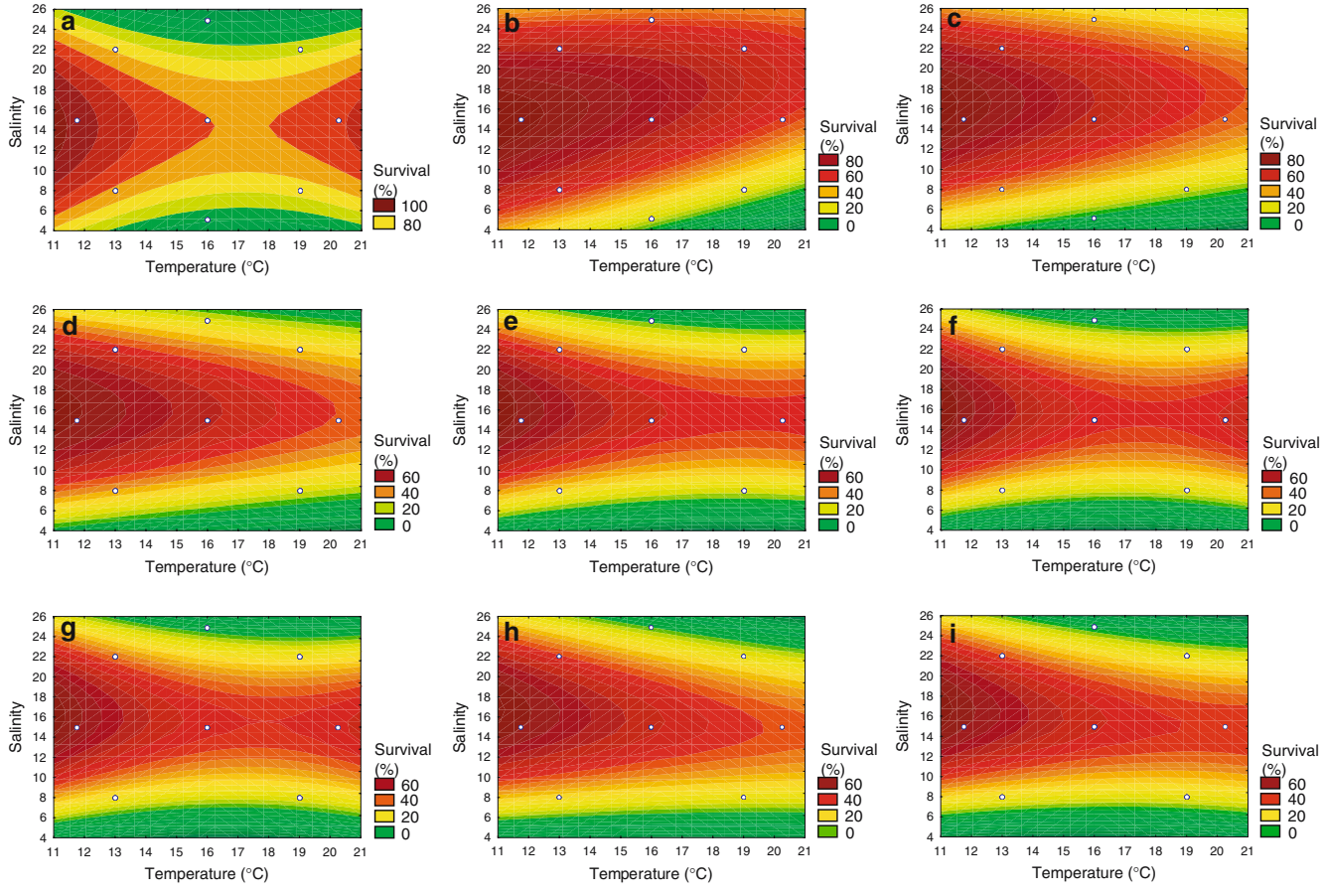

Fig. 4 Response profiles of the survival (%) at age 4 (**a**) to 12 (**i**) days

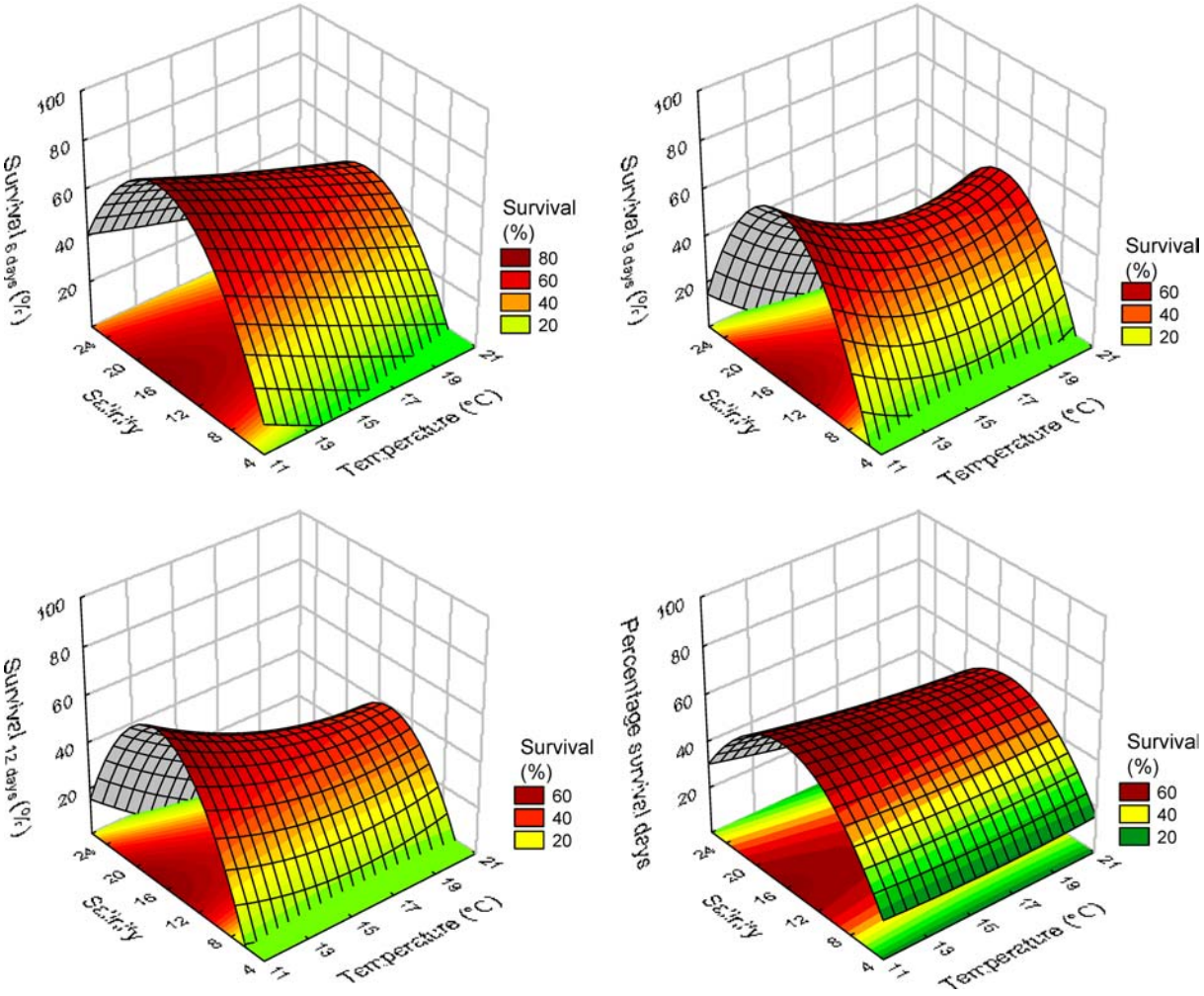

Fig. 5 Response surface plot of the fitted models for survival at age 6 days (**a**), 9 days (**b**), 12 days (**c**) and percentage survival days (**d**)

the dependent variables (percentage survival at a certain day, percentage survival days, hatching percentage, duration of stages I, II, III and total development time and standard length of the offspring) were used to fit a response surface model including the linear and quadratic main effect and the two-way interactive effects (STATISTICA 6.0). The significance of the effects (including the factor 'block') was tested with an ANOVA. Since in each block the control treatments were replicated, the pure (random) error could be quantified and the residual variance tested using a Lack of Fit test.

Results

Description of the intra-marsupial development

The intra-marsupial development of *N. integer* was divided into 3 substages in the present study, while generally for mysids a subdivision into 3 to 12 substages is common (Berrill 1969; Mauchline 1973; de Kruif 1977; Wittmann 1981). Table 2 and Fig. 2 summarize the terminology used by the different authors, including the one used in the present study, and applied to the observed morphology in the intra-marsupial development of *N. integer* (with supporting pictures).

The early embryos (stage I) are spherical or subspherical (Fig. 2a). Rudiments of antennae and abdomen are developing (Fig. 2b) and observable under low magnification (25×) as a lighter-coloured disk. The abdominal rudiment is ventrally bent and develops anteriorly towards the cephalic appendix. Stage I ends with the hatching from the egg membrane by puncturing it with the developing abdomen. The shed egg membrane quickly disintegrates, but is sometimes visible in the wells.

The stage II larvae are dorsally bent and have a comma-like appearance. Initially, a rudimentary abdomen with a clear distinction between the rounded anterior and the pointed posterior of the larva can be observed together with two thoracic appendages (Fig. 2c). In a later phase the abdomen shows the clear beginning of segmentation, however without any appearance of appendages (Fig. 2d). Later on, the body is further extended and the thoracic appendages more

Table 4 Regression coefficients [± standard error (SE)] of the response surface model fitted to the survival at age 3, 6 and 9 days and the percentage survival days with their *P* values; ANOVA effect estimates with their *P* values for the fitted model including the Lack of Fit test

		Regression coefficient	SE	*P* value	ANOVA		R^2
					F value	*P* value	
Survival age 6 days	Intercept	26.469	101.974	ns			
	T	−6.287	11.570	ns	15.927	< 0.001	
	T^2	0.032	0.349	ns	0.008	ns	
	S	13.582	3.483	< 0.001	21.329	< 0.001	
	S^2	−0.451	0.064	< 0.001	50.139	< 0.001	0.461
	T×S	0.108	0.177	ns	0.374	ns	
	Block				33.956	< 0.001	
	Lack of fit				4.585	< 0.001	
Survival age 9 days	Intercept	49.773	73.703	ns			
	T	−19.084	10.987	ns	5.822	< 0.05	
	T^2	0.594	0.334	ns	3.154	ns	
	S	21.207	3.754	< 0.001	3.437	ns	
	S^2	−0.609	0.077	< 0.001	63.021	< 0.001	0.493
	T×S	−0.129	0.163	ns	0.629	ns	
	Block				17.595	< 0.001	
	Lack of fit				1.221	ns	
Survival age 12 days	Intercept	−49.215	97.690	ns			
	T	−7.927	12.108	ns	6.664	< 0.05	
	T^2	0.290	0.379	ns	0.585	ns	
	S	22.810	3.636	< 0.001	3.268	ns	
	S^2	−0.597	0.076	< 0.001	62.142	< 0.001	0.519
	T×S	−0.255	0.155	ns	2.699	ns	
	Block				18.404	< 0.001	
	Lack of fit				1.660	ns	
Percentage survival days	Intercept	−6.125	73.541	ns			
	T	−1.749	8.344	ns	1.865	ns	
	T^2	−0.061	0.252	ns	0.058	ns	
	S	10.771	2.512	< 0.001	0.735	ns	
	S^2	−0.312	0.046	< 0.001	46.182	< 0.0001	0.446
	T×S	−0.073	0.128	ns	0.329	ns	
	Block				61.390	< 0.0001	
	Lack of fit				1.383	ns	

T temperature; *S* salinity; *ns* not significant

Table 5 Regression coefficients [± standard error (SE)] of the response surface model fitted to the hatching percentage with their *P* values; ANOVA effect estimates with their *P* values for the fitted model including the Lack of Fit test

		Regression coefficient	SE	*P* value	ANOVA		R^2
					F value	*P* value	
Hatching percentage	Intercept	−78.913	73.546	ns			
	T	0.069	8.344	ns	10.296	< 0.01	
	T^2	0.014	0.252	ns	0.003	ns	
	S	17.519	2.512	< 0.001	2.194	ns	
	S^2	−0.477	0.046	< 0.001	108.010	< 0.001	0.524
	T×S	−0.174	0.128	ns	1.865	ns	
	Block				25.794	< 0.001	
	Lack of fit				0.855	ns	

T temperature; *S* salinity; *ns* not significant

elongated (Fig. 2e). The larvae have globules of the yolk protein vitellin within their tissues. These globules are homogeneously distributed throughout the body in stage I embryos and early stage II larvae, but as the yolk volume decreases relative to the body volume, the yolk becomes more concentrated in the anterior dorsal regions at the end of stage II. Dorsally the optical rudiment is visible as an anterior cleft (Fig. 2f). As the larva grows, the naupliar cuticle is stretched and the uropods and telson are formed. Eight abdominal segments are clearly visible. Lateral chromatophores appear, mainly in the anterior part (Fig. 2g). The optical lobes are visible with pigmented eye rudiments (Fig. 2h). A rhythmic beating of the heart and contractions of the gut are visible. The naupliar stage II terminates with the moulting from the naupliar cuticle.

The post-naupliar stage III larvae (Fig. 2i) have stalked eyes, a developed telson and uropods without

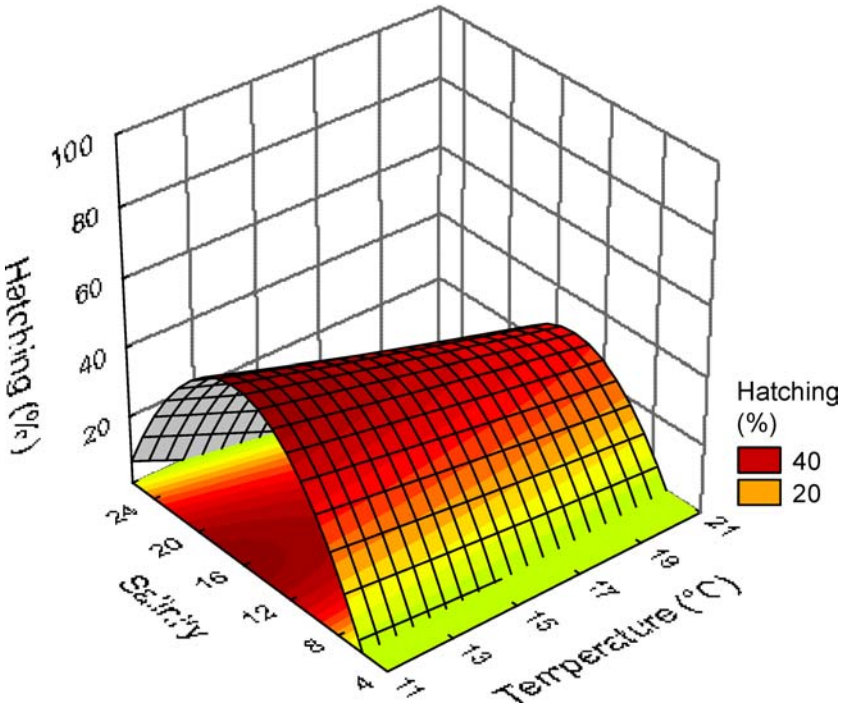

Fig. 6 Response surface plot of the fitted model for hatching success

lith in the statocyst of the inner ramus. The thoracic appendages, mouth parts and antennae are developing. All over the body, darkly pigmented chromatophores appear. Close to the end of this stage a carapace can be observed (Fig. 2j). The larvae are very actively moving by a longitudinal dorsal flexing and stretching of the body. Also an active rhythmic moving of the thoracic appendages is observed. Stage III terminates in a moult, leading to free-living young juveniles (Fig. 2k) that are, except for the sexual characteristics, morphologically similar to the adults. The gradually disintegrating yolk is completely consumed.

Short-term survival experiment

The mean percentage survival of the stage I embryos after 72 h at all tested salinity and temperature combinations is shown in Table 3. At all temperatures tested, the survival was $\leq 10\%$ at salinity 2.5, and $\leq 20\%$ at salinity 5.0. At the other salinities, survival was substantially higher and was maximal at 15 and 16°C (64%). These results motivated the selection of the temperature range (13–19°C, centred at 16°C) and the salinity range (8–22, centred at 15) used in the Central Composite Design (Table 1).

Table 6 Mean duration of the embryonic development and substages [in days, ±standard error (SE)] for all treatments of the Central Composite Design

Treatment	Temperature (°C)	Salinity (psu)	Total	Stage I	Stage II	Stage III
A	16.0	15.0	15.3 ± 0.2	5.0 ± 0.3	7.0 ± 0.0	3.6 ± 0.2
F	16.0	15.0	15.8 ± 0.2	4.4 ± 0.2	7.4 ± 0.2	3.2 ± 0.2
I	16.0	15.0	15.8 ± 0.1	5.3 ± 0.3	7.2 ± 0.1	3.8 ± 0.1
J	16.0	15.0	16.2 ± 0.1	4.8 ± 0.2	7.8 ± 0.2	3.6 ± 0.2
Average control	16.0	15.0	15.8 ± 0.1	4.9 ± 0.2	7.4 ± 0.1	3.6 ± 0.1
B	13.0	8.0	18.0 ± 0.0	5.0 ± 0.3	9.5 ± 0.5	4.0 ± 0.0
C	19.0	22.0	12.0 ± 0.0	4.8 ± 0.2	4.4 ± 0.2	2.8 ± 0.3
D	13.0	22.0	19.7 ± 0.2	5.5 ± 0.3	9.3 ± 0.2	4.2 ± 0.2
E	19.0	8.0	12.0 ± 0.0	4.0 ± 0.0	5.0 ± 0.0	3.0 ± 0.0
G	16.0	5.1	–	–	–	–
H	16.0	24.9	–	6.8 ± 0.7	–	–
K	11.7	15.0	21.8 ± 0.1	5.8 ± 0.2	10.7 ± 0.1	5.3 ± 0.1
L	20.2	15.0	11.2 ± 0.1	4.3 ± 0.2	4.2 ± 0.1	2.4 ± 0.2

– Data excluded from analysis

Table 7 Regression coefficients [± standard error (SE)] of the response surface model fitted to the total development time and the duration of stages I, II and III with their *P* values; the ANOVA effect estimates with their *P* values for the fitted model including the Lack of Fit test

		Regression coefficient	SE	*P* value	ANOVA		R^2
					F value	*P* value	
Total development time	Intercept	37.098	2.272	< 0.001			
	T	−1.967	0.246	< 0.001	3989.548	< 0.001	
	T^2	0.028	0.007	< 0.001	16.002	< 0.001	
	S	0.501	0.153	< 0.01	10.703	< 0.01	
	S^2	−0.008	0.004	< 0.05	4.202	< 0.05	0.984
	T×S	−0.012	0.006	ns	3.804	ns	
	Block				13.779	< 0.001	
	Lack of fit				4.596	< 0.05	
Duration stage I	Intercept	6.833	3.730	ns			
	T	0.094	0.417	ns	24.190	< 0.001	
	T^2	−0.010	0.012	ns	0.608	ns	
	S	−0.231	0.180	ns	4.720	< 0.05	0.354
	S^2	0.008	0.004	< 0.05	4.120	< 0.05	
	T×S	0.003	0.009	ns	0.110	ns	
	Block				7.459	< 0.01	
	Lack of fit				1.381	ns	
Duration stage II	Intercept	16.987	2.584	< 0.001			
	T	−0.492	0.283	ns	1151.915	< 0.001	
	T^2	−0.005	0.008	ns	0.441	ns	
	S	0.085	0.173	ns	1.523	ns	
	S^2	−0.0002	0.005	ns	0.002	ns	0.951
	T×S	−0.007	0.007	ns	0.998	ns	
	Block				5.459	< 0.05	
	Lack of fit				0.124	ns	
Duration stage III	Intercept	10.586	2.959	< 0.001			
	T	−0.591	0.321	ns	156.135	< 0.001	
	T^2	0.006	0.009	ns	0.479	ns	
	S	0.029	0.199	ns	0.085	ns	
	S^2	−0.003	0.005	ns	0.367	ns	0.701
	T×S	0.005	0.008	ns	0.324	ns	
	Block				1.498	ns	
	Lack of fit				3.851	ns	

T temperature; *S* salinity; *ns* not significant)

Survival

The cumulative survival functions were plotted as percentage survival (Fig. 3). Table 1 shows the percentage survival days and the median survival time of the 12 treatments. The percentage survival days of treatment F was significantly lower than in the other control treatments ($P < 0.0001$). However a two-factor ANOVA (treatment × age) of the percentage survival did not show a significant interactive effect ($P = 0.960$). For comparative purposes the survival of the four centre points were therefore treated as equal and plotted as the average percentage survival (grey line in Fig. 3).

In the treatments G (salinity 5.1) and H (salinity 24.9) at 16°C, an extreme high mortality was noted and all embryos/larvae had died at the age of 6 and 13 days, respectively. Note that treatment G has salinity and temperature conditions that were identical to the conditions in which the adults were kept and mating took place. At 19°C, both at salinity 8 (E) and 22 (C), a high initial mortality occurred. However some larvae in a low number of replicates did survive and hatched (respectively 6 and 29%). At 13°C a higher survival was observed at the higher salinity (salinity 22, D) in comparison with the low salinity treatment (salinity 8; B). In general, the highest mortality occurred within the first 6 days of the embryonic development, i.e. during stage I. An ANOVA demonstrated significant differences in the percentage survival days within all treatments ($P < 0.0001$). A significantly higher mean value ($> 55\%$ day^{-1}) of the percentage survival days was observed at salinity 15 at temperatures between 11.7 and 20.2°C (centre points, K and L). This is reflected in the higher median percentage survival (> 10 days).

Figure 4a–i plots the response profiles of the age-specific survival at day 4 through day 12 (beyond this latter later age the dataset becomes too incomplete as a result of hatching or mortality). The number of temperature and salinity combinations with the highest survival ($> 60\%$, indicated in the darkest colours) decreases with increasing age. Figure 5 shows the response surface of the survival at age 6, 9 and 12 days and the percentage survival days. Highest survival is associated with the lowest temperatures (< 14°C) and medium

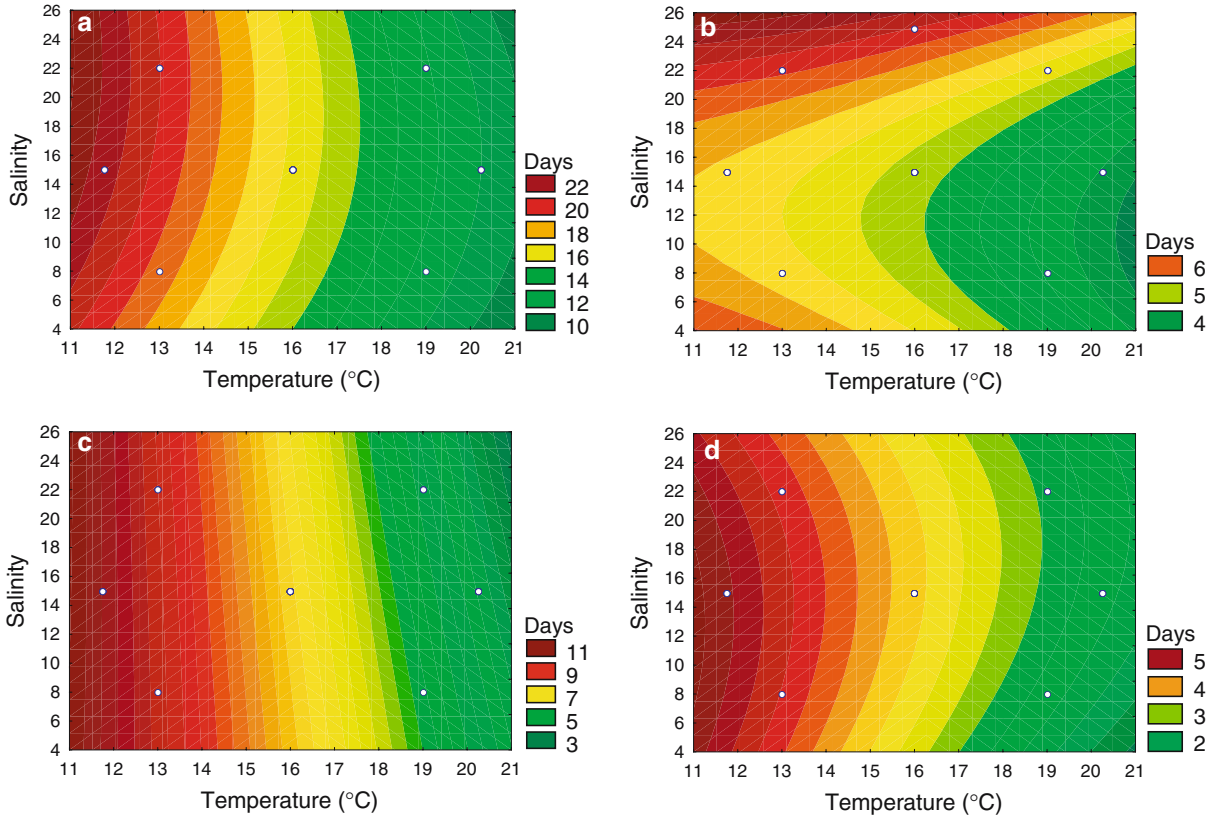

Fig. 7 Response profiles for the total development time (**a**), and duration of stage I (**b**), stage II (**c**) and stage III (**d**)

salinities (salinity 12–22). The regression coefficients and the ANOVA effect estimates (Table 4) indicate a relatively poor fit of the response surface models ($R^2 = \pm 0.5$). Note the highly significant effect of the block ($P < 0.0001$). For the survival at day 6 significant variation is observed which is due to unexplainable error. Despite the relatively poor fit, the models indicate that the survival is mainly affected by the quadratic salinity effect and to a lesser degree by the linear effect of salinity and temperature (especially at the beginning of the incubation period).

Hatching success

The centre point treatments at 16°C and salinity 15 (A, F, I and J) do not differ in their hatching success (ANOVA, $P = 0.569$) and are further treated as one (Table 1): 79% of the control treatments replica resulted in hatching and 49.5 ± 3.6% of the initial embryos lead to free-living juveniles. Note that in treatment G and H no hatching was observed due to complete mortality. Significant differences in the hatching percentage between all treatments were noted ($P = 0.010$). This is mainly due to the significant lower hatching in the C treatment (19°C–salinity 22) of only 16% of the larvae. Hatching at a salinity of 15 is significantly affected by temperature as demonstrated by a hatching success at 11.7°C and 20.2°C of 57% (K) and 36% (L), respectively.

Regression coefficients and the ANOVA results of the response surface modelling indicate a poor fit (Table 5, Fig. 6). The hatching success is significantly affected by the quadratic salinity effects and the linear temperature effect. Hatching percentage is highest at the moderate salinity (± 16 psu) and low temperature (< 15°C) combinations (Fig. 6). Hatching success is 10–20% lower at higher temperatures (> 15°C).

Duration of development

The duration of the intra-marsupial development varied between 11 and 22 days, of which stage I took on average 31% of the time, stage II 45% and stage III 23%. Again the centre point treatments were further considered as one (Table 6). Due to the extreme poor survival in treatments G and H, these data were excluded from the analyses. ANOVA's revealed significant differences between the treatments for the stages I, II and III, and the total duration of the development (all at least with $P < 0.001$). In general, the duration of the intra-marsupial development decreased with increasing temperature as demonstrated in the 15 psu treatments: at 20.2°C (L), 16°C (Control) and 11.7°C (K), total development duration was 11.2, 15.8 and 21.8 days,

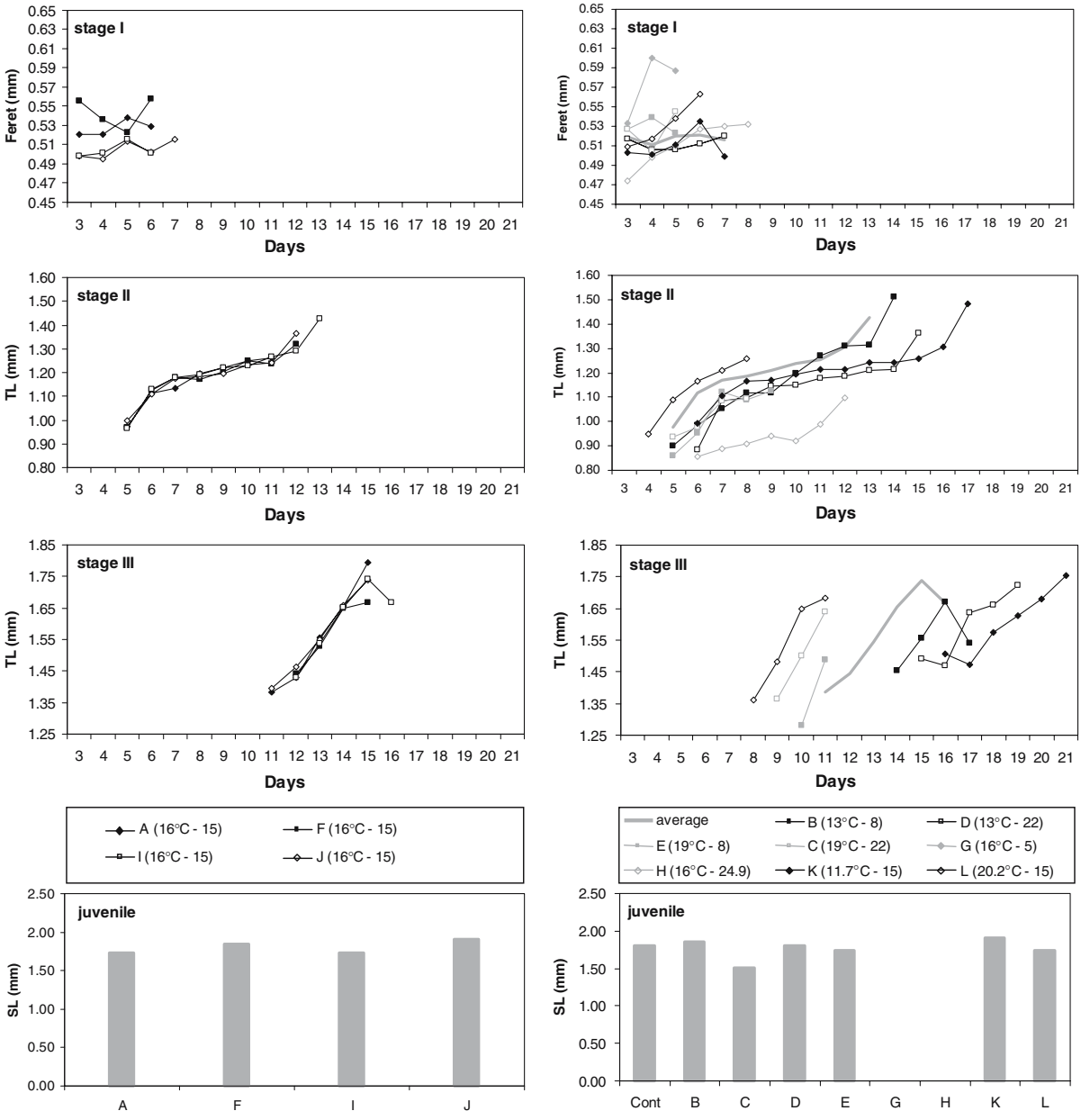

Fig. 8 Embryo size related to age of the four centre point treatments (*left*); Embryo size related to age in all treatments (temperature°C–salinity) of the central composite design with the mean of the centre point treatments in *grey* (*right*). Standard errors are generally smaller than 0.05 mm. (*TL* total length; *SL* standard length)

respectively. This is mainly due to a reduction of stage II with increasing temperature from 11 to 4 days (61%) at the lowest and highest tested temperature, while stage I reduces on average from 6 to 4 days (26%) and stage III from 5 to 2 days (55%).

The response surface models (Table 7 and Fig. 7) revealed a good fit for the total development time ($R^2 = 0.984$) and the duration of stage II ($R^2 = 0.951$) and III ($R^2 = 0.701$). It is mainly the linear temperature component that controls the duration of the intra-marsupial development, although salinity also has some minor influence in the observed patterns (Table 7). Note that the factor 'block' also had a significant effect.

Size and growth rate of the embryos and larvae

Stage I

The Feret's diameter of the stage I embryos (Fig. 8) was tested within the two subsequently performed blocks, taking age into account. Although the two-factor ANOVA indicated that there was no significant interactive effect (Feret × age; $P = 0.071$), the size of early embryos in block 1 was always larger than the embryos that had contributed to the second block ($P < 0.0001$). Age does not have an effect on the Feret's diameter of the spherical embryos ($P > 0.05$). The stage I embryos

Table 8 Growth rate in stages I, II and III of all treatments of the Central Composite Design

Treatment	Temperature (°C)	Salinity	Stage I (Feret's diameter)		Stage II (Total length)		Stage III (Total length)	
			%	mm day^{-1}	%	mm day^{-1}	%	mm day^{-1}
A	16.0	15.0	2	0.003	30	0.05	30	0.10
F	16.0	15.0	0	0.001	36	0.05	16	0.07
I	16.0	15.0	4	0.004	37	0.05	25	0.09
J	16.0	15.0	1	0.001	48	0.06	17	0.06
Average control	16.0	15.0	0.4	0.0005	46	0.06	20	0.06
B	13.0	8.0	1	0.001	68	0.07	6	0.03
C	19.0	22.0	3	0.009	17	0.05	20	0.14
D	13.0	22.0	1	0.001	54	0.05	16	0.06
E	19.0	8.0	−0.8	−0.002	31	0.07	16	0.21
G	16.0	5.1	10	0.027	–	–	–	–
H	16.0	24.9	12	0.012	28	0.04	–	–
K	11.7	15.0	−0.7	−0.001	50	0.04	16	0.05
L	20.2	15.0	11	0.018	33	0.08	24	0.11

did not measurably increase in size (Table 8), except for those in the treatments L, G and H. The latter salinity–temperature combinations did exhibit high mortality. Probably the size increase of >10% in the egg-like embryos is an indication of a near dead.

The centre point treatments of the second block (I and J) resulted in embryos with smaller size than those in the treatments covering centre points A and F of the first block ($P=0.0001$). However, differences were also observed among centre point treatments within one block, with the F embryos being larger than the A ones (Fig. 8). Despite the randomization protocol used in the test design, the initial size of the embryos was not identical in all treatments. To avoid misinterpretation of the data, the relative growth (in % day^{-1}) was used as an endpoint to evaluate the size of stage I embryos between the 12 treatments, assuming this parameter is not affected by the initial size discrepancy. From this analysis it appears that the mean size of stage I embryos of one replica is either increasing or decreasing (up to +15 to −15% day^{-1}), indicating that the Feret's diameter is an unsuitable size measure of stage I embryos.

Stage II

The length of stage II larvae of a certain age did not significantly differ between the control treatments A, F, I and J (ANOVA; $P=0.370$) and are further treated as one control (Fig. 8). Stage II larvae increase in size substantially with time ($P=0.0001$; Table 8); compared to their initial size an increase of 46% was noted. At the lowest temperatures (B, D, K) the growth of stage II larvae was higher (50–68%) than that in treatments C, E and L at 19 and 20.2°C (17–32%). However, at lower temperatures the development lasted longer, resulting in a rather uniform growth rate between the treatments (0.04–0.08 mm day^{-1}). The larvae at the highest salinity of (24.9 psu; H) did not survive stage II and a suboptimal growth was observed.

Stage III

The total length of stage III larvae at a certain age was not different between all control treatments ($P=0.635$). A significant increase in size (20%) as a function of time

Table 9 Regression coefficients [±standard error (SE)] of the response surface model fitted to the standard length (SL) of the hatched offspring with their *P* values; ANOVA effect estimates with their *P* values for the fitted model including the Lack of Fit test

		Regression coefficient	SE	*P* value	ANOVA		R^2
					F value	*P* value	
Hatching size (SL)	Intercept	1.430	0.558	ns			
	T	0.028	0.014	<0.05	26.457	<0.001	
	T^2	−0.000	0.050	ns	0.000	ns	
	S	0.051	0.001	ns	3.145	ns	
	S^2	−0.000	0.001	ns	0.139	ns	0.138
	T×S	−0.003	0.002	ns	3.308	ns	
	Block				5.755	<0.05	
	Lack of fit				0.929	ns	

T temperature; *S* salinity; *ns* not significant

was observed ($P < 0.0001$). Again temperature had a pronounced effect on the size of the stage III larvae. However, here we noted that lower temperatures (B, D and K) reduce the growth rate (0.03–0.06 mm day^{-1}) in comparison with the warmer temperatures (C, E and L; 0.11–0.21 mm day^{-1}).

Juveniles

The free-living juveniles in treatments A and I were significantly smaller than those in F and J ($P < 0.0001$). Also in the comparison with the other treatments, some differences are observed (ANOVA, $P < 0.0001$), but no trend with salinity or temperature could be demonstrated. The fit of the response surface within the central composite design is low (Table 9), and only temperature and the factor 'block' had an adverse effect on the standard length of the hatching offspring.

Discussion

Methodology

The eggs were extruded from the oviducts into the marsupium and closely packed together in two (a left and right) packages, which are enclosed together with the sperm within a very thin membrane (as described by Kinne 1955 as tertiary egg membranes). Since the jagged-shaped embryos do not have a firm consistency yet, removal of the eggs from these membranes is impossible without damaging. This is the reason why the in vitro experiment starts 3 days after deposition in the marsupium, when the thin membranes are dissolved and the embryos are spherical and firm. An additional advantage is that unfertilized eggs, disintegrating within 24 h, are not included into the bioassays.

In a preliminary experiment, improved survival and a shorter development time were observed when the multiwell plates were placed on an orbital shaking table (80 rpm). The continuous movement on a roller table probably simulates the rhythmic lateral moving of the brood lamellae by the gravid female to irrigate and provide oxygen to the embryos/larvae and positively affects the hatching success and shortens the development time (Mauchline 1980; Fernández et al. 2002). This phenomenon was especially apparent in stage III as the oxygen demand of crustacean embryos/larvae increases as they develop (Smith and Klieber 1950; Fernández et al. 2003).

Number and size of embryos and larvae in the marsupium

The number of embryos/larvae in the marsupium of female mysids depends upon the body size of the female, the size of the individual eggs, the season of the year and geographic location (latitude) of the mysid populations (Mauchline 1980). For *N. integer* the brood size is demonstrated to be highly depended on female body size, which varies seasonally (Kinne 1955; Parker and West 1979; Mees et al. 1994): late summer- and autumn-breeding animals usually have a smaller size-at-maturity compared to those breeding in spring and early summer. The size of the early embryos also varies seasonally (for *N. integer*: Mauchline 1973; Irvine et al. 1995), with winter embryos being larger than in spring or summer.

The initial size of the stage I embryos from block 1 and 2 (from day 3 to days 6–7) was clearly different, with the block 1 embryos always being significantly larger. Due to logistic limitations two subsequent blocks were set off, with embryos from females caught, respectively between 16/03/2004 and 30/03/2004 and between 6/04/2004 and 20/04/2004. The water temperature had increased from 9.8 to 12.2°C during this period. *N. integer* has a growth stop in winter time (Mees et al. 1994). From the moment water temperature rises above 10°C, growth and development is triggered. Larger (spring) animals have a higher fecundity and the size of the embryos is negatively related with the brood size (Mauchline 1973). This may explain the difference in the initial size of the two blocks used in our design. The observed size difference clearly had an impact on the other measured endpoints (as demonstrated by a significant effect of 'block' in the ANOVA's while fitting the response surface models). For future experiments, it is recommended to use *N. integer* from a continuous and well-standardized laboratory culture. If this is not possible we advise to avoid working with different blocks in the design, or to keep the time difference between the blocks to a minimum. The size of newly released juveniles was significantly different between treatments. Reduced size or weight at birth may have implications for the future survival and breeding potential of the offspring (Kolding and Fenchel 1981; Wehrtmann and Lopez 2003).

The size and growth of the embryos and larvae do not seem to be useful for evaluating the influence of environmental variables on the intra-marsupial development of *N. integer*. This seems to be especially true for the ellipsoid stage I embryos. Variation on the measurement of the Feret's diameter is high because of the ellipsoid form. However, Wittmann (1981) and Lawrence and Poulter (2001) found the maximal width and length of ovaline-shaped embryos to be a good endpoint. The sub-spherical embryos were measured with a larger accuracy (light microscope) than the current stereomicroscope measurements (50×). The stage I embryos of treatments G and H showed a significant increase in the Feret's diameter. This was probably due to osmotic swelling of the embryos prior to their eventual disintegration (Morritt and Spicer 1996b).

Intra-marsupial mortality—Hatching success

The hatching success was highest (max. 58%) in treatments with an intermediate salinity of 14–17 psu

combined with a low temperature (< 15°C). Especially the earliest stages (first 6 days) are susceptible to unfavourable salinity and temperature conditions. The mortality of the embryos and larvae during the in vitro experiment ranged from 40 to 100%. Mauchline (1973) reports a marsupial mortality of about 12–13% for *N. integer*, while Irvine et al. (1995) estimated intra-marsupial mortality of *N. integer* to be in excess of 50%, and mainly occurring at the beginning of the development. In the field, mortality might also be caused at the end of the development by accidental loss of late stage II and III larvae, when gravid females have distended marsupial lamellae to contain the large larvae and move the marsupial lamellae to irrigate the larvae (Mauchline 1980).

Intra-marsupial development time

The duration of the incubation period is a key factor in the understanding of the population biology of a species and is related to the timing of the breeding season, age at maturity, frequency of broods, number of young per brood, egg size and adult body size (Wittmann 1984). Intra-marsupial development time, i.e. from appearance in the brood pouch to the release of young, is highly related to the environmental temperature (Mauchline 1980; Wittmann 1984) and not or little affected by salinity (Vlasblom and Elgershuizen 1977; Greenwood et al. 1989). In the present experiment the development time varied respectively between 22 and 10 days at temperatures between 11 and 21°C, respectively. Irvine et al. (1995) provide indirect estimates of the intra-marsupial development time of *N. integer* from Hickling Broad, UK (salinity ± 3): 56 days (at 7–16.5°C), 29 days (at 16.5–18°C), and 13–14 days (at 19.5–20°C). From their experiments, the same authors concluded that the development time was 42 days at 6°C and 6 days at 20°C at a salinity of 3. The latter development time is extremely short in comparison with the present results and the authors' field-derived estimate at the same temperature. Kinne (1955) performed laboratory experiments at 10 psu aimed at establishing the total intra-marsupial development time of *N. integer* and reports 20 days at 11°C and 14–15 days at 19°C. Vlasblom and Elgershuizen (1977) obtained (experimentally) a constant duration of 16–18 days at varying salinities (0.4–16 psu) at 15°C. Taking the age-at-maturity into account (N. Fockedey et al., in press), the intra-marsupial development of *N. integer* takes 15–17% of the generation time at 20°C and 16–21% of the generation time at 15°C (respectively at 15 and 5 psu). These values are considered as typical for temperate mysid species (Wittmann 1984).

Stage II is mainly responsible for the prolongation of the total development time at lower temperatures. At the extremes of the temperatures stage II is delayed from 4 days at 21°C to 11 days at 11°C. The growth of larval structures is possible through the conversion of egg proteins, mainly vitellin. A delay in stage II indicates that temperature has a strong influence on the developmental processes and thus on egg protein metabolism. Recently, Ghekiere et al. (2004) purified and characterized vitellin from *N. integer* with the aim to develop an enzyme-linked immunosorbent assay (ELISA) to quantify the yolk protein. Future laboratory and field studies will evaluate the use of this immunoassay for investigating effects of abiotic variables and xenobiotics on *N. integer* vitellogenesis.

In general, a prolongation of the intra-marsupial development time may reduce survival by prolonging a sensitive and vulnerable life stage. In Mysidacea, this is compensated for by the protection provided by the intra-marsupial development of the organisms. Indeed, at lower temperatures (combined with a medium salinity) the final survival and hatching success is even better. We found the experimental salinity to have a minor impact on the duration of larval development of *N. integer*. Earlier findings of Vlasblom and Elgershuizen (1977) indicate that the experimental salinity does not influence the intra-marsupial development, but that animals adapted to a higher salinity generally take longer time to develop. In the present study, this adaptation factor was not taken into account (all animals originated from a salinity of ± 5).

All the larvae within a single marsupium are at the same stage of development (Mauchline 1980). The occasional presence of younger larvae among a brood is usually attributed to adoption (Wittmann 1978; Mauchline 1980). However, in our experiments some variation in the time of transition form one stage to another occurred and may explain these observations. A 1 or 2 days delay in transition from stage I to stage II occurred in 48 and 18% of the organisms. In the moulting of stage II to stage III or from stage III to the juvenile, a 1 day delay occurred in 21 and 14% of the cases.

Salinity optimum

The salinity range at which the embryos and larvae develop is more restricted than the salinity range at which the female mysids can survive (Vlasblom and Elgershuizen 1977; Greenwood et al. 1989). Although fertilization in the laboratory occurred at a salinity of 5 and a temperature of 16°C, the embryos cultured in vitro at this salinity and temperature combination (G) never developed to free-living juveniles. Complete mortality occurred after 6 days. Vlasblom and Elgershuizen (1977) found a survival of 0–30% of the early embryos at a comparable salinity.

In the subtidal of Gironde, Weser, Tamar and Schelde estuaries (Sorbe 1980; Schuchardt et al. 1989; Moffat 1996; N. Fockedey, unpublished), ovigerous females mainly occur in the low salinity zone (salinity 3–10), indicating that no migration from the adverse salinity conditions occurs in *N. integer*. Also, permanent

populations are described in enclosed low-saline brackish ponds and lakes (e.g. Irvine et al. 1995). Our experimental (in vitro) results on the intra-marsupial survival and hatching success of *N. integer* at this low salinity suggest that the recruitment success of juveniles to the population may thus be seriously affected, unless the gravid female is able to actively regulate the salinity within its marsupium and in this way increase the survival and hatching success of its offspring. The active regulation of the marsupial fluid salinity is described for isopods (Charmantier and Charmantier-Daures 1994), amphipods (among others Morritt and Spicer 1996b) and mysids including *N. integer* (Ralph 1965; McLusky and Heard 1971), although the technique of freezing point analysis used by the latter authors has been criticized by Morritt and Spicer (1996b). The measurement of haemolymph and marsupial fluid concentration of *N. integer* over a range of salinities with more modern techniques (e.g. direct-reading nanolitre osmometer) is required to confirm or reject the hypothesis of active marsupial salinity regulation for the species.

Also the mechanism for the (possible) regulation of the marsupial fluid salinity in mysids remains unknown and needs further study. A pair of tubes extending ventrally from the female's thorax into the marsupium is described for *N. integer* (Vorstman 1951; Kinne 1955). Although their function is unknown (Mauchline 1980), they may have a secreting function (Kinne 1955) and may hence have a role in the regulation of the ionic composition of the marsupial fluid. Morritt and Spicer (1996b) suggested that the marsupial salinity can be actively regulated, as described for amphipods, by redirecting urine from the antennary excretory gland into the brood pouch.

Population differences

Genetically different populations of a species may differ in the way their intra-marsupial development is affected by salinity (Lee 1999) and temperature (Wittmann 1984). The in vitro development time of embryos of *N. integer* individuals adapted to higher salinities (salinity 23) is longer than that of embryos taken from a low salinity (salinity 7) population (Vlasblom and Elgershuizen 1977). The *N. integer* individuals used in the present experiment were sampled from a dock along the Schelde estuary. Although the dock is connected to the estuary, population genetic analysis based on mitochondrial cytochrome oxidase I sequences revealed this population to be significantly distinct from the population living in the subtidal of the Schelde estuary (T. Remerie et al., submitted) or other estuaries (T. Remerie et al., submitted). The responses to temperature and salinity differences reported in this paper may be population-dependent and need further study.

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