Food mediated life history strategies in *Daphnia magna*: their relevance to ecotoxicological evaluations

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Food mediated life history strategies in *Daphnia magna*: their relevance to ecotoxicological evaluations

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Stellingen

1 Het kweken van *Daphnia*'s bij hoge voedselconcentraties leidt tot produktie van kleine nakomelingen die slecht bestand zijn tegen voedselschaarste en toxische stoffen. Het gebruik van deze nakomelingen in acute testen zonder voedsel kan gezien worden als een worst case-benadering.
- dit proefschrift

2 Alleen als een laboratorium goede reproduktietesten kan uitvoeren, is het in staat om ook voor acute testen proefdieren van constante kwaliteit te leveren. Standaardisatie van voedselgiften is hiervoor noodzakelijk.
- L. Viganò, *Wat. Res.* 27 (1993) 903-909.

3 Toepassing van het von Bertalanffy type I groeimodel in populatiedynamische *Daphnia*-modellen leidt tot de voorspelling dat lichaamslengten van concurrerende cohorten convergeren, hetgeen niet in overeenstemming is met waarnemingen.
- dit proefschrift

4 Extrapolatiemodellen die onvoldoende rekening houden met de fenotypische plasticiteit en individuele variatie van organismen kunnen geen adequate voorspelling geven van de effecten van een toxicant op populatieniveau.
- dit proefschrift

5 Het is een misverstand te menen dat populatieëxperimenten de geëigende manier zijn om toxische effecten op populatieniveau te bepalen. Veelal kan worden volstaan met eenvoudiger proeven met individuen. Tenzij voldoende inzicht bestaat in alle overige bepalende factoren en onderliggende biologische mechanismen zijn experimenten met populaties pure bezigheidstherapie.

6 Er is geen reden te veronderstellen dat de toetsconcentratie waarbij geen statistisch significant effect wordt waargenomen (NOEC) meestal lager is dan de geschatte concentratie waarbij een relatief klein effect, bijvoorbeeld 5% (EC_5), optreedt.

7 De EROD-methode is een uitstekend instrument om temperatuur te meten.

8 De effecten op onze kustwateren van de gereduceerde nutriëntenaanvoer door de rivieren worden gemaskeerd door een verhoging van de gehalten in het ontvangende oceaanwater.

9 De grootste bijdrage die een ecotoxicoloog aan de milieukwaliteit kan leveren is het beperken van zijn eigen emissies en (die van) zijn nageslacht.

10 Diffuse verontreiniging is een probleem van iedereen en daardoor van niemand.

11 Natuurbehoud is een contradictio in terminis, natuurontwikkeling een pleonasme.

12 Gemak doodt de mens.
- J. Enserink

Stellingen behorende bij het proefschrift 'Food mediated life history strategies in *Daphnia magna*: their relevance to ecotoxicological evaluations' door Lisette Enserink, te verdedigen op maandag 11 december 1995 te Wageningen.

Vanuit de nevels
roeiden wij; hoe breed lag toen
de zee daarachter.

Shiki

Voor mijn moeder,
ter herinnering aan mijn vader en Tessa

Contents

chapter 1

Introduction

Context of the current study

The research presented in this thesis was carried out at the Institute for Inland Water Management and Waste Water Treatment (RIZA), which is a research and advisory institute of Rijkswaterstaat (Directorate General of Public Works and Water Management). Its advices, covering the whole field of freshwater management, are used by the main water authorities. The results of its investigations are of national and international significance. RIZA has been involved in ecotoxicological research since 1979.

In cooperation with TNO Institute of Environmental Sciences and the Agricultural University at Wageningen the project 'Effects of pollutants on *Daphnia* populations' has been conducted between 1987 and 1991. The main purpose of this project was to relate effects on individual *Daphnia* to effects on populations. The present study pertains to interactions between life history strategies and toxic substances, which are related to extrapolation and standardisation of toxicity test results.

Daphnia in aquatic ecotoxicology

The use of ecotoxicology in water quality management has gained increasing attention in recent years. For instance, Dutch water quality standards are based on ecotoxicological data since 1989 (Ministerie van Verkeer en Waterstaat, 1989). Also, within the framework of the European Community, toxicity tests have been adopted as a screening method to assess toxicity of dangerous substances in the aquatic environment (EC, 1990). As yet, incorporation of bioassays in waste water licences and sanitation of contaminated sediments is not widespread. However, water management organisations are beginning to acknowledge the surplus value of ecotoxicological data, i.e. information on bioavailability of toxicants and effects of complex mixtures and unknown substances.

A limited number of organisms is used in routine toxicity tests. Among these, crustaceans of the genus *Daphnia* probably are the oldest (Naumann, 1934) and most widely used test organisms. They are regarded as important representatives of filter-feeding zooplankton that play a distinct role in many freshwater ecosystems. Moreover, they are relatively easy to culture and laboratory populations normally consist of only one genotype, owing to parthenogenetic reproduction under non-stressed conditions. Therefore, *Daphnia* has been recognized as an important tool for the management of water quality during the last decades.

With increasing government interest more emphasis has been put on the development of relatively simple laboratory tests, among which acute and chronic studies with *Daphnia magna* Straus play a prominent role.Toxicity tests should be both predictive and reproducible in order to

indicate safe concentrations of chemicals in the aquatic environment and to obtain reliable parameters for regulatory purposes respectively.

The predictive potential of a given set of test results is determined by the resemblance of laboratory and field conditions, that modify the sensitivity of the test organism. There are indications that genetic and environmental factors have considerable effects on the outcome of toxicity tests. However, very few systematic studies have been dedicated to this subject (Chandini, 1989; Persoone *et al.*, 1989; Baird *et al.*, 1991).

Reproducibility of test results, i.e. the homogeneity of the results of repeated tests, is improved by standardisation of test methods and culture techniques (Adema, 1978; Buikema *et al.*, 1980; Cowgill, 1987). Throughout the years of toxicity testing evidence has accumulated that test results depend on the test method as well as the culture technique. However, present knowledge still is insufficient for the development of adequately standardized test guidelines. It is clear that both predictability and reproducibility benefit from knowledge of the reaction of *Daphnia* to its environment, with reference to the laboratory practice of toxicity testing.

Effects of environmental factors, for instance food and temperature, on feeding, metabolism, growth and reproduction have been extensively studied on the level of the individual (Green, 1956; Hebert, 1978; Lynch, 1980; Lampert 1987). The 'life history' approach, which is common in ecology, can help to substantiate the prefix 'eco' in ecotoxicology. According to Threlkeld (1987) 'a life history is a pattern of age-specific allocations of available (ingested and assimilated) resources to reproduction or to non-reproductive efforts that may affect changes of future reproduction'. In this definition food availability plays a key role.

Feeding and reproduction in *Daphnia*

Quality and availability of food are predominant factors in the physiology and ecology of animals. This explains that the subject has received considerable attention in studies regarding feeding behaviour, assimilation, respiration, allocation of energy and competition for food. A thorough review has been published by Lampert (1987).

Daphnia, like many other Cladoceran species, can be characterized as particle feeders. The feeding apparatus of *Daphnia* consists of thoracic appendages, which carry large, filter-like screens. Together with the carapace they pump water, containing food particles, from head to tail through the gape of the carapace. Particles retained by the screens are transported to the ventral food groove and moved towards the mouth. Here, the food is grinded by the mandibles before egestion takes place (Lampert, 1987). *Daphnia* is a relatively passive filter feeder. However, unwanted food, for instance filamentuous algae or excess food, can be removed from the food groove by the postabdominal claws or rejected by the mouthparts (Richman & Dodson, 1983). The maximum size of particles entering the carapace is limited by the distance between the edges of the carapace valves. The size of the gape is narrowed in dense algal suspensions, thereby preventing obstruction of the filtering apparatus. This mechanism may increase survival during summer algal blooms (Gliwicz & Siedlar, 1980). The mesh size of the filtering apparatus is about 1 μm in *D. magna* (Porter *et al.*, 1983). Both mesh size and the area of the filtration screen increase with body size (Egloff & Palmer, 1971).

The ingestion rate of *Daphnia* increases proportionally to food concentration until it reaches a plateau (Rigler, 1961), denoted as the incipient limiting level. Below this concentration *Daphnia* exhibits its maximum filtering rate, above it the maximum ingestion rate is found. At very low food concentrations a slight depression of the filtering rate has been observed, probably due to exhaustion (Muck & Lampert, 1980). Many authors have measured the shape of the curve relating food concentration to ingestion rate. Due to biological and methodological differences a large variation between the results for one species can exist (Kasprzak *et al.*, 1986).

The rate of assimilation, which is the second step in the process of energy uptake, depends on the same factors that control the ingestion rate. Therefore, the functional response with respect to the concentration of food is similar to that of the ingestion rate (Lampert, 1987). Other factors affecting these rates are body size, temperature, diel rhythms and light.

Compared to, for example, calanoid copepods, *Daphnia* has a relatively large, energy expensive feeding apparatus. Besides, inappropriate food is rejected after it has been collected, due to aselective filtration. Therefore, feeding is inefficient at both low and high food densities. In the first case a large volume of water has to be filtered to collect a small quantity of food. In the second case more food is collected than can be ingested. For these reasons Richman & Dodson (1983) characterized *Daphnia* as a taxon most suitable for a habitat in which food quality is high and the abundance of food is neither extremely high nor low.

A characteristic behaviour of *Daphnia* is vertical migration induced by the intensity of light (Ringelberg, 1987). At night *Daphnia* feeds on algae in the epilimnion, during the day she stays in the hypolimnion to digest or feed on non-algal food (Dini *et al.*, 1987). The biological relevance of this behaviour might be to escape visual predators, e.g. fish.

Nannoplanktonic algae normally are the main food source for *Daphnia* (Lampert, 1987). Besides, grazing on bacteria (Hadas *et al.*, 1982), some blue-green algae (Holm *et al.*, 1983), ciliate protozoans (Porter *et al.*, 1979) and detritus (Tóth *et al.*, 1987) has been reported. The nutritive value of food, which depends on ingestibility, assimilability and biochemical composition, can only be measured in growth experiments (Lampert, 1987).

The life cycle of *D. magna* depends on environmental conditions (Green, 1956; Hebert, 1978). Under constant, non-stressed conditions, which are pursued by test laboratoria, it can be summarised as follows. At temperature of *c.* 20 °C, a daily photoperiod of approximately 12 h and abundant food eggs develop in the brood pouch in *c.* 3 days. Neonates are released as free-swimming miniature adults. After a juvenile period of 7 to 10 days, in which the animal moults 5 or 6 times, the first brood is born. Succeeding broods are then released every 3 days approximately, which is followed by a moult shortly thereafter. Body size can only increase before the new carapace is hardened, which results in 'saltatory' growth. In a single female three broods can develop simultaneously. One clutch of eggs develops in the brood pouch, while the second clutch is provisioned in the ovaries. At the same time a third clutch differentiates from nurse cells to oocytes (Zaffagnini, 1987). Growth and reproduction can continue until senescence.

Standardisation of culture methods

While attributes of *Daphnia*, e.g. size, generation time, genetic homogeneity and ready fecundity, are desirable for laboratory work, the many uncertainties surrounding basic requirements for the organism's maintenance often interfere substantially with experimentation. Among the most common problems are those related to establishing a combination of diet and culture medium, which is satisfactory in terms of culture health and reproducibility of test results. Several investigators have searched for defined laboratory diets (Schwartz & Ballinger, 1980; Cowgill *et al.*, 1985; Keating & Dagbusan, 1986; Winner, 1989). From these studies it was learned that a stable multigeneration reproduction is the only proof for the qualitative and quantitative adequacy of a diet (Keating & Dagbusan, 1986).

The importance of culture methods for the results of toxicity tests has been indicated by a few studies. Cowgill *et al.* (1984) found a relationship between maternal conditions and the fitness of the progeny. In addition, evidence was obtained that the results of acute tests are affected by the condition of the neonates (Baird *et al.*, 1989). Nevertheless, current international guidelines, for instance OECD (1984), apply to maintenance of test animals, whereas only a brief outline of culturing conditions is given. Obviously, more research is needed to assess the relative importance of maternal conditions to the outcome of toxicity tests.

Extrapolation of test results

From the results of single-species toxicity tests ecotoxicological protection levels are derived. Extrapolation from laboratory to field is commonly based on no observed effect concentrations (NOECs) of preferably chronic toxicity tests with different species. The maximum tolerable risk concentration (MTR) of the chemical under consideration is derived from combined NOECs using a statistical model, or from the lowest NOEC, which is devided by a safety factor.

A more mechanistic approach for the first step of extrapolation, i.e. from the individual organism to a single-species population, was developed in the project 'Effects of pollutants on *Daphnia* populations'. Models for individual growth and reproduction (Kooijman & Metz, 1984; Kooijman, 1986), which are based on the physiology of *Daphnia*, were used for the prediction of population dynamics (Kooijman *et al.*, 1989; Van der Hoeven, 1991). In this way, toxic effects on individuals can be extrapolated to the population level. Studies with laboratory populations of *D. magna* were carried out to test the predictions of the model (Van der Hoeven, 1990). However, there was some dissimilarity between model predictions and experimental results. For instance, density oscillations in computer simulations were more pronounced than in laboratory populations. This result was related to assumptions regarding the influence of food level on two major life history traits, i.e. size at maturation and body growth.

More theoretical *Daphnia* models have been developed in recent years (Nisbet *et al.*, 1989; Fitsch, 1990; Hallam *et al.*, 1990), but some of their assumptions still await experimental validation. Assumed relationships between food level and life history parameters, e.g. size at birth, age and size at maturation, body growth, instar duration, number of progeny, etc., are crucial to the results of

model simulations. Especially, more insight into aspects of growth and reproduction at low food is required.

The experimental approach: outline of the thesis

In the present thesis food availability to *D. magna* takes a central position. The importance of food for the culture of test animals is explored, as a contribution to standardisation of toxicity tests. In order to support development of extrapolation methods the model of Kooijman (1986; Kooijman *et al.*, 1989), which stimulates new hypotheses and new observations, is investigated. The current study validates assumed relationships between food level and *Daphnia* growth and reproduction. It focuses on two major life history traits in particular, i.e. size and fitness of newborn young, and age and size at maturation.

Cadmium, chromium and lead, which are designated as priority pollutants by the International Rhine Committee (IRC, 1987), were used as model substances to investigate the interactions between toxicants and life history mechanisms.

The experimental approach has been chosen to investigate both toxicity test- and model-based questions. Experiments were carried out at different levels of integration, i.e. the individual and population level. For studies with individual *D. magna* simple experimental set-ups were appropriate, but special equipment was designed for the experiment with populations. Detailed descriptions are given in *chapter 7*.

A general description of RIZA culture methods is presented in *chapter 2*. This part also reports the development of an automatic sizing technique using computerized image analysis.

Chapters 3 and 4 describe the consequences of food quantity in *Daphnia* cultures for the quality of the progeny, with emphasis on their sensitivity in acute (*chapter 3*) and chronic (*chapter 4*) toxicity tests. Cadmium and chromium were used as model compounds. In addition, the relationship between brood size and neonate size under different feeding conditions, i.e. the reproductive strategy, was explored (*chapter 3*). The ecological significance of this strategy is further elaborated in *chapter 4*. The results are compared with assumptions of Kooijman's *Daphnia* model (1986): size at birth is independent of food level and neonate energy reserves are proportional to maternal reserves (*chapter 7*).

In this model it is assumed that size at maturation is independent of food level, with important consequences for the dynamics of simulated populations. In *chapter 5* the influence of food availability on age and size at maturation is evaluated. Growth and ovary development of individual *Daphnia* were followed at a broad range of food rations. The partition of biomass between growth and reproduction is compared with a fourth model assumption, i.e. the fraction of energy allocated to reproduction is independent of food level, except for starvation conditions. In toxicity tests time to first reproduction is measured on a routine basis, but the ecotoxicological relevance of effects on maturation has received only little attention. In this chapter combined effects of food level and lead are evaluated.

Convergence of body sizes and synchronisation of life cycles, being the main cause of unrealistic density oscillations in population simulations, was studied in competition experiments (*chapter 6*).

A culture system was developed to simulate food dynamics and competition during a density peak and succeeding decline phase of a laboratory population. Growth and maturation in two competing cohorts of different age were investigated. The application of empirical growth curves in *Daphnia* models is discussed, with reference to the growth model assumed by Kooijman (1986).

Chapter 7 gives a general discussion and concluding remarks regarding the ecological and ecotoxicological relevance of the results.

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chapter 2

The experimental approach: culture methods and automatic length measurements

Culture methods*

Introduction

Standardisation of methods for the culture of *Daphnia magna* has received growing attention in recent years. It has become increasingly clear that the past history of test animals can influence the results of ecotoxicological experiments. Nevertheless, current international guidelines, for instance EC (1986), only give a brief outline of the method. Research laboratories are responsible for development of detailed protocols. Hence, each laboratory uses a unique culture protocol, which is mainly based on local experience. Advanced standardisation of these protocols would improve inter-laboratory reproducibility. However, the freedom of a laboratory to adapt the culture method to local circumstances is thus restricted, which is the main objection against standardisation.

In this chapter a comprehensive description of the methods currently practised at the Institute for Inland Water Management and Waste Water Treatment is given, as an example of 'proven - not yet standardized - technology'.

Daphnia culture

The *D. magna* population used in our laboratory originates from a single female by means of parthenogenetic reproduction. In The Netherlands most ecotoxicological research institutes appeared to use the same genetically homogeneous clone, as could be concluded from a study on isozyme patterns in several clones (Baird *et al.*, 1991). It was named 'clone 4' by the investigators.

All experiments in this thesis were carried out with *D. magna* cultured in natural water from Lake IJssel, a large eutrophic lake. The composition of this medium may change with the seasons, in particular the levels of total organic carbon (Rijkswaterstaat & RIVM, 1985). Small particles and dissolved compounds may influence the nutritive state of *D. magna* cultures. Besides, toxic excretions of blue-green algae, which bloom during summer months, may threaten culture health. Indeed, summer times often coincided with a decline of fitness and production. In concordance with an international standardisation programme, coordinated by the OECD, our laboratory switched to the artificial M4 medium (Elendt, 1990) in May 1991. It has been successfully used ever since.

The natural medium, which was used in the present studies, originated from a location near Lelystad. A volume of 300-400 l was transported to the laboratory once or twice a month. To remove zooplankton the water was filtered through 50 µm plankton gauze. It was kept in a covered

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polyethene tank at 20 ± 1 °C and aerated continuously with 0.25 µm filtered, compressed air. Prior to use the water was pumped along a pair of lamps emitting ultraviolet light (Wedeco, type M2-6), filtered through 25 µm gauze and stored in a 50 l polyethene tank, supplied with continuous aeration. Two or three times a week a new quantity was prepared. The tank was wiped clean regularly. Table 1 gives the average analysis of Lake IJssel water.

Table 1.
Composition of Lake IJssel medium for *D. magna* culture.

chemical		concentration (mg/l)
trace elements	Fe	0.015
	Mn	0.089
	Cu	0.0026
	Zn	0.004
	Ni	0.0029
	Pb	0.0001
	Si	0.4
macronutrients	Ca	72
	Mg	11
	Na	60
	K	7
TOC		4.5
hardness*		225

*as $CaCO_3$ (based on Mg and Ca)

Our *D. magna* culture consisted of 4 to 8 cohorts of *c.* 60 individuals each. A cohort was maintained for 4 to 5 weeks. Once or twice a week a new cohort, consisting of *c.* 80 neonates (<24 h) from clutch number ≥3 of a common mother cohort, was separated. Parentage and date of birth were recorded. The cohort was thinned down to 60 females once eggs appeared in the brood pouch. Mortality could further decrease the size of the cohort.

The culture was kept in a temperature controlled room (20 ± 1 °C) with a 12 h photoperiod of moderate intensity. Cohorts were maintained in covered full-glass aquaria containing 5 l of medium, which was aerated continuously. Every day a food ration of 5×10^9 *Chlorella pyrenoidosa* cells (34 mg TOC) was added to each aquarium; this was given in halves (early morning and late afternoon) on working days. On Monday, Wednesday and Friday the animals were transferred to fresh medium. Any newborn young and dead animals were removed and the latter were also recorded. For each aquarium a clean glass tube (⌀ 5 mm) with a rubber teat was used. Used glassware, i.e. aquaria and pipettes, was cleaned in a laboratory washing machine to minimize the risc of cross-infection.

Normally, only parthenogenetic reproduction occurred under these conditions. Reproduction within a cohort was synchronised, which enabled determination of brood number and prediction of time of birth. Mean mortality was well below 10% during the first 3 weeks (Fig. 1). Superfluous animals were fed to fish cultures.

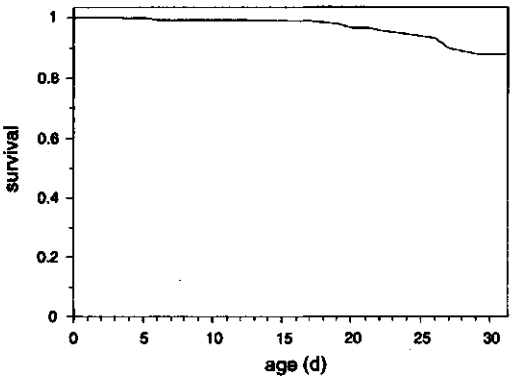

Fig. 1.
Survival in *D. magna* culture. The average survival of 33 cohorts during 6 months is presented.

Occasionally, males or ephippial eggs were found. These were removed from the aquaria. Cohorts with more than 20% males were eliminated, because of questionable quality of the young. Progeny of cohorts with lower male frequencies were excluded from chronic toxicity tests and stock culture, though they were used in acute tests if nescessary. The same applies to offspring of the grandmother cohort.

In case of mould infestations, affected cohorts were eliminated and extra care was taken to prevent exchange of water between cohorts.

Chlorella culture

The laboratory diet of *D. magna* consisted of *C. pyrenoidosa*, an unicellular green alga. This alga was batch-cultured under axenic conditions, using the medium described in Table 2. All glassware was autoclaved and culture procedures were carried out under sterile conditions. The temperature of the culture was 20 ± 1 °C. As a long-term stock a pure culture was kept on agar plate, which was prepared from the algal medium. Twice a year it was transferred to fresh agar. From this stock 100 ml pre-cultures were started, which were grown in Erlenmeyer flasks, stoppered with non-absorbent cotton wool. During 4 weeks the flasks were kept in an orbital incubator and illuminated at 100 $\mu E/(m^2 \times s)$ (daylight colour). After 9 days the suspension was ready to use for inoculation of large cultures. Portions of *c.* 7.5×10^7 cells were added to screw cap bottles, supplied with a septum, which contained 2.5 l of medium. Twice a week two (or more) batch cultures were started. They were intensively aerated with 0.25 µm filtered, compressed air and illuminated at 300 $\mu E/(m^2 \times s)$. Daily the bottles were shaken thoroughly to resuspend sedimentated algae. After 7 days cell density

Table 2.
Composition of medium for *C. pyrenoidosa* culture.

chemical		added as	concentration (mg/l)
trace elements	B	H_3BO_3	0.254
	Mn	$MnCl_2.4H_2O$	0.253
	Zn	$ZnSO_4.7H_2O$	0.0251
	Mo	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0049
	Cu	$CuSO_4.5H_2O$	0.010
	Co	$Co(NO_3)_2.6H_2O$	0.050
	Fe	$NH_4Fe(SO_4)_2.12H_2O$	0.060
	EDTA	$Na_2EDTA.2H_2O$	0.32
macronutrients	KNO_3		1250
	$CaCl_2$		7.5
	$MgSo_4$		147
	K_2HPO_4		287
	KH_2PO_4		185

was *c.* 5×10^{10} cells/l, cell volume was *c.* 16 μm^3 and population growth still was exponential. The bottles were disconnected from the air supply and cooled down to 5 °C in a dark room to stop cell multiplication. After one day the algae were concentrated to a volume of 250 ml by centrifugation (30 min., 4500 rpm) at 5 °C. Cell density was checked with a Coulter Counter (Coulter Electronics; Harpenden, England). Concentrated algae were stored at 5 °C and used to feed the *D. magna* cultures for a maximum period of 2 weeks. Regularly, the purity of the pre-cultures was checked with a microscope.

In the present study food rations are given as the number of algal cells. The mean carbon content of a *C. pyrenoidosa* cell is 7×10^{-6} µg C in our laboratory.

Automatic sizing with image analysis*

Introduction

Plankton research usually is bound up with counting, sizing and identification of individual specimen. Traditionally, plankton samples are elaborated by the human eye, with the aid of microscope, micrometer and a simple counting chamber. As this is a rather arduous, time-consuming task methods have been developed to automatize the process. The widely used Coulter Counter technique for instance, has proven its suitability for counting and sizing of small, regularly shaped particles, e.g. algal suspensions. For zooplankton samples computerized image analysis appears to be a more promising method. It combines rapidity of electronic counting with visual control of microcopical analysis. Basic parameters of an object, i.e. length, width, area and perimeter, can be used for the identification of taxonomic groups (Jeffries *et al.*, 1980, 1984) and the construction of a size frequency distribution (Rolke & Lenz, 1984).

Within the scope of this thesis computer-aided determination of body length in large samples of living *D. magna* was pursued. For this purpose a computer program was developed, which was based on a multi-purpose image processing system called TIM (Ekkers, 1990).

System description

The TIM program by TEA (Dordrecht, The Netherlands) and Difa Measuring Systems B.V. (Breda, The Netherlands) runs on a MS-DOS computer (IBM-PC and compatibles). The hardware further consists of the following elements (Fig. 2):

- a black and white CCD-video camera (High Technology Holland, type MO, 50 ;
- a PCVISIONplus frame grabber (Imaging Technology Inc., type PFGplus-512-3-E-AT);
- a colour monitor for image display (Sony, type PVM 1371 QM).

The camera was mounted on an adjustable camera stand for magnification and focusing. A sample of *Daphnia* (50 individuals at the maximum) in a watch glass was placed under the camera. Excess water was removed with a pipette in order to reduce swimming movements. As *Daphnia* is highly transparant, it was illuminated from the sides by a six-point glass fiber ring-light (⌀ 60 mm) connected to a cold light illuminator (Euromex, type EK-I). The ring-light's radiance was scattered by white, semi-transparant 3 mm perspex. Black paper below the ring-light provided for a dark background. The combination of dark background and diffuse illumination enlightened the carapace and improved contrast at its edges (Fig. 3). Our experimental set-up is depicted in Fig. 4.

The video image produced by the camera was digitized by the frame grabber and send to both computer and image display monitor. This monitor is also connected to the computer in order to display the stages of image processing.

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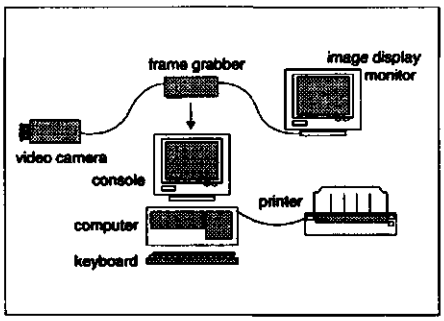

Fig. 2.
TIM hardware components (from: Ekkers, 1990).

Fig. 3.
Camera view of the experimental set-up. The circle is just included in the video image.

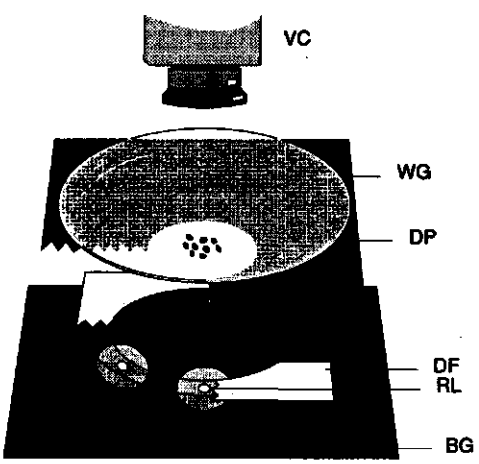

Fig. 4.
Schematic representation of the experimental set-up. VC video camera; WG watch glass; DP diaphragm; DF diffusing milk-white perspex; RL six-point ring-light; BG background.

Image processing

A digitized image consists of a matrix of 512×512 picture elements, or pixels. In the computer's memory each pixel is represented by 8 bits. With this memory capacity a pixel can store one grey value from a set of 256 values, where 0 is black and 255 is white. A grey value image can be turned into a binary image by thresholding. Pixels with values greater than or equal to the threshold grey value will turn white (value 1) and pixels with lower values will become black (value 0). Only one bit per pixel is needed to store this information. For local operations subimages can be defined within the 512×512 image. Images can reside in the frame grabber memory, in the standard computer memory, or in a file. Filed images offer the possibility of batch-processing of a series of images, a method used to speed up the acquisition of images.

Most preparations for length measurement are binary operations that modify the shape of the objects. By convention, object pixels are indicated with 1 and background pixels with 0. Pixels belong to the same object if they are connected in one of 8 directions (N, NE, E, SE, S, SW, W and NW).

For correct length measurements of *Daphnia* several modifications in the original video image are nescessary. A computer program was written to execute the procedure described below. It uses two images stored in the frame grabber memory and one image stored in a file.

For the calibration of length measurements a multiplication factor is calculated from the number of pixels between two points on a ruler. The ruler is placed in the same position to the camera as a sample containing *Daphnia*. The calibration factor is stored in a memory buffer.

The video image of a *Daphnia* sample containes light objects against a dark background. Ideally, the background should be composed of pixels in a small range of (low) grey values. In practice, spatial inhomogeneities of the illumination cause brightness gradients. In order to correct for background inhomogeneity, an 'empty' image, i.e. without objects, is subtracted from the image to be processed (Fig. 5, top). The empty background image is stored in a file for multiple use.

In the next step, the grey level image (Y) is converted into a binary image by a threshold operation using a pre-set grey value. In low-contrast objects, e.g. *Daphnia*, the threshold value affects the size of the object. Therefore, a routine was developed to compare the size of thresholded objects to the original image visually. Both threshold value and illumination were standardized.

In order to remove small particles, e.g. clumps of algae and pseudo-faeces, the edge pixels of the objects are removed by a single erosion. This action takes place in binary image 2, while a copy of the original shape is kept in binary image 1. In case no pixels are left, the object is lost. Remaining objects are grown to their original shape by a propagation (Fig. 5, bottom): the 'seeds' in binary image 2 can only grow if the new pixels already were present in the 'mask' binary image 1.

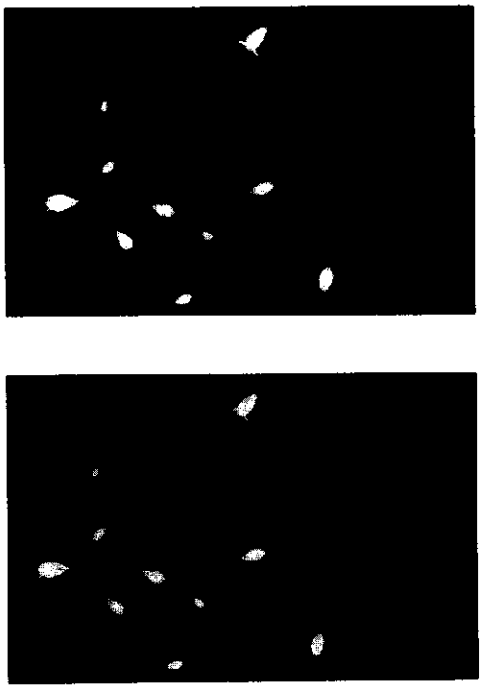

Fig. 5.
Monitor display of whole image operations. Top: grey value image after background correction. Bottom: binary image after erosion and propagation. Small objects have been removed.

Some objects may touch, or even cross, the border of the image. Since this may lead to erroneous length measurements such objects are removed. Therefore, a white border is drawn in the outer pixels of the image to connect them. In the next pass all objects, including the compound 'object', are labelled with a unique grey value. In this process the image is scanned from the upper left corner to the lower right corner. The computer first encounters the border of the image and removes the compound object. The remaining objects are given successive grey values and the number of objects is recorded.

The subsequent series of operations is performed in subimages just enclosing a single object. The series is repeated until all objects in the image have been measured. The operations are shown on the display monitor in a colour overlay on top of the original grey value image (X), that serves as a visual reference. The thresholded subimages are copied from image Y into 3 binary images, each presented by a different colour. The whole process is illustrated in Table 3.

Table 3.

Modification of object shape in subimages of X.

Operation	binary image 3	2	1
Erase binary images 3, 2 and 1			
Write object, indicated by a specified label value, from image Y to binary image 3			
Single erosion in binary image 3 to remove antennae			
Single dilation in binary image 3 to restore object size			
Invert binary image 3			
Draw a white border around the subimage in binary images 3 and 1			
Single propagation, starting from the border, in binary image 1 with binary image 3 serving as a mask. Holes are removed.			
Invert binary image 1			
Copy binary image 1 to binary image 3			
Remove alle object pixels, except the contour pixels			
Skeletonize the contour to produce an 8-connected chain of pixels			
Find contour pixels with greatest mutual distance and draw a line between them in binary image 2			

An erosion is used to remove antennae and caudal spines. The size of the body is restored by a dilation, expanding each object pixel with its background neighbours. As a result of partial transparency of *Daphnia* some parts of the body can be lost during thresholding. If this problem concerns the carapace edge, restoration of the animals's shape is not possible. However, holes can be repaired by a propagation, starting from the border of the subimage in a seed binary image. An inverted image of the object in another binary image serves as a mask. The propagation stops if it encounters the contour pixels of the object. An inversion of the seed binary image yields the improved object shape.

The final length measurement uses the contour pixels of the object, described by a Freeman chain code (Freeman, 1970). The maximum distance between pixels in a closed contour is estimated using the corner count method (Vossepoel & Smeulders, 1982). This distance is indicated on the display monitor by a green line in the object and it is written to a file.

Performance of the method

In the present set-up pixel size was 0.055×0.055 mm^2. This implied that particles smaller than 0.11 mm were removed by erosion. Occasionally, larger contaminants remained in the image. Experimentation learned that measured object lengths less than 0.55 mm could be removed from the results file, without losing small neonates (body length *c.* 0.7 mm).

Measurements on living zooplankton suffer from rapid movements and frequent encounters of two or more animals. In addition, the orientation of free swimming *Daphnia* can yield biased length determinations. A convenient solution was to reduce the amount of water, which demobilises the animals and enables lateral measurements. Animals that touch each other could be separated manually. Quick, yet careful action is required in order to minimize exposure time to air and injury owing to handling. Except for moulting individuals, no indication of enhanced mortality was found. The incidence of blurred images owing to rapid movements of *Daphnia* can be further reduced by a high frequency camera or a high speed shutter.

The precision of automatic length measurement was compared to manual measurements of *D. magna*, carried out with a zoom stereo microscope. Repeated measurements of the same individuals, with a length range from 1 to 3 mm, showed that image analyser results were less precise than microscope determinations: coefficients of variation were 9% and 1% respectively. Lengths of small juveniles were slightly (*c.* 8%) overestimated by the image analyser, compared to the microscope. Substantial differences however, could occur by measuring large females. The illumination technique used for image analysis was less suitable for these animals, yielding large grey value differences within the body. Mostly, the caudal part of the carapace, which is highly transparent, was lost owing to thresholding (Fig. 6). The large antennae often survived erosion, thus increasing object size. Hence, body lengths of large adults could be under- or overestimated.

Fig. 6.
Measurement of a gravid female. *A.* original thresholded object. *B.* object contour after modifications. *C.* maximum length. Arrow indicates antennae.

The main advantages of automatic sizing are speed and comfort. A single image could contain up to 50 animals. Five to 10 minutes were needed to prepare the video image and computer processing took only a few seconds. Thus, a single person can measure *c.* 2000 living animals per day, which is hardly attainable using a microscope.

Image analyser or microscope?

Automatic image analysis is a more rapid, but less precise method for length measurements than the classical combination of microscope and human eye. Hence, the method of choice depends on the research question.

If, for instance, length frequency distributions of *Daphnia* populations are to be determined, image analysis is the best choice. Within a population, body lengths range from neonate to full-grown adult. Individuals in the lower and upper tails of the distribution may be relatively rare, but they yield essential information; i.e. size at birth and maximum adult size respectively. Fig. 7 shows that the lowest and highest size classes comprise only a few percent of the population. Thus, representative samples should contain at least 50 to 100 animals. In this case rapid measurement with image analysis is advantageous.

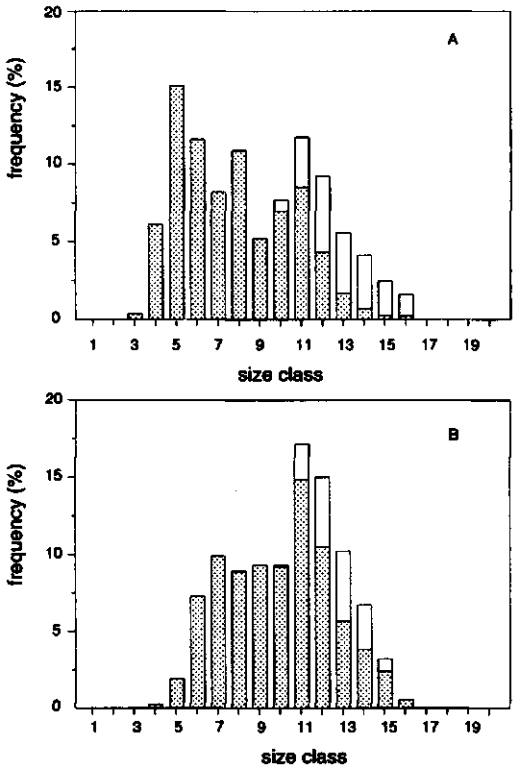

Fig. 7.
Size frequency distributions of *D. magna* populations determined with image analysis. Population *A* (n=778) is adapted to high food levels and *B* (n=689) to low food. Class width is 0.221 mm; lower limit of class 1 is 0 mm. Empty bars: females carrying eggs; shaded bars: females without eggs. Unpublished data from a study with expanding populations according to Smith's method (Smith, 1963).

However, for determinations of individual growth curves a high precision is required. Length increase between instars ranges from 1% to 40%, depending on body size and food availability. Growth rates less than 10% are found in reproducing adults and at low food levels (cf. chapter 5). As the coefficient of variation is 1% for microscope and 9% for image analyser, it will be clear that measurements by microscope are the best choice.

In the current study precision of length measurements was more important than large numbers and speed. Hence, the zoom stereo microscope was used in most of the experiments..

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chapter 3

Reproductive strategy of *Daphnia magna* affects the sensitivity of its progeny in acute toxicity tests*

Abstract

Maternal nutrition in *Daphnia magna* affects the body size of the young and the sensitivity of the brood for acute exposure to cadmium, but not to chromium(VI). This phenomenon may contribute to inter- as well as intra-laboratory variation in test results, because *Daphnia* culture techniques are not yet standardized in a sufficient way.

In one set of trials four food ration levels of the green alga *Chlorella pyrenoidosa* were given to a cohort of adult *D. magna*. After eight days brood size and the size of young became adapted to the new food ration level. At low levels, small broods were produced which consisted of large neonates, whereas at high food levels many tiny young were born.

In acute toxicity tests with cadmium the sensitivity of the neonates appeared to be connected with their body size. The smallest young were three times more sensitive than the largest neonates, comparing the 48-h LC_{50}. No difference in sensitivity occurred during acute exposure to chromium.

Introduction

The study was carried out to investigate whether the reproductive strategy of *Daphnia magna* affects the vulnerability of their offspring to toxic chemicals. The sensitivity of young daphnids used in toxicity tests may be connected with the reproductive activity in *Daphnia* cultures. A number of studies showed that brood size and the size of neonates vary considerably according to the feeding regime. Smith (1963) for instance noticed that neonates from adult *D. magna* in rapidly growing laboratory populations were on the average smaller in size than newborn found in slowly growing, food limited populations. Cowgill *et al.* (1985) showed that different types of diets (e.g. several species of algae, fish food) also markedly influenced the neonate size as well as the brood size of *D. magna*. The largest broods consisted of lighter neonates than those produced in smaller broods. They suggested the existence of a reproductive strategy. Females will spread their genes around by producing many 'cheap' neonates under favourable conditions, while a small number of heavy, stress-resistant young are born when food is sparse.

However, there is some controversy. Tessier *et al.* (1983) demonstrated that adult *D. magna* in low food environments transfer less maternal lipid to each egg than animals in high food environ-

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ments. Their results also showed a positive correlation between lipid content of neonates and their survival time in the absence of food.

In the present experiment, which was based on the results of a pilot study (Enserink, 1989), the reproductive response of *D. magna* to food ration levels was re-examined. A sudden change in food level was applied to evaluate the flexibility of the reproductive strategy. The consequenses with respect to the sensitivity of the progeny in acute toxicity tests are shown.

Materials and methods

Experimental conditions

Experiments with *Daphnia magna* from our laboratory stock were carried out at 20 ± 1 °C with a 12 hour photoperiod. The test medium used was 25 μm filtered, UV-treated Lake IJssel water with a pH of 8.1 and a hardness of approximately 225 mg/l (as $CaCO_3$). Daphnids were fed batch-cultured green algae (*Chlorella pyrenoidosa*). In order to obtain a stable food quality, the algae were harvested in the exponential growth phase and stored in a dark room at 5 °C for a maximum period of four days. Daphnids were held in glass vessels containing 1 l of medium or in small jars with 50 ml of medium. The number of cells in newly prepared medium was checked by means of a Coulter Counter. The water was replaced daily.

Feeding studies

Three different experimental set-ups were used simultaneously, which varied according to the number of animals per volume of water and to the vessel size. Daphnids were kept with 10 individuals/l (experiment A), 20 ind/l (experiment B) and 1 individuals/50 ml (experiment C) respectively. The animals were given constant daily rations of *C. pyrenoidosa* until their third brood was born, the individuals in experiment A receiving twice as many algal cells (5.6×10^7/-(ind×d)) as those in experiments B and C (2.8×10^7/(ind×d)). On day 16, each group was subdivided into four subgroups, each receiving a different food level: 1.4×10^7, 2.8×10^7, 5.6×10^7 or 11.2×10^7 algal cells/(ind×d) (Fig. 1).

At the start of the experiment daphnids (<24 h) were randomly distributed among 12 vessels in experiments A and B, and 40 small beakers in experiment C. Newborn daphnids were counted daily and discarded. At all brood numbers, ca. 50 neonates (<4 h) were sampled from each (sub-)group. Carapace length and lipid index (Tessier & Goulden, 1982) were measured microscopically. Weekly video pictures of living individuals of the parent generation were made to measure their carapace length in a non-destructive way. The experiment was terminated after 32 days. The resistance to xenobiotics of young from the seventh brood of the parent generation was examined by exposing offspring to two heavy metals.

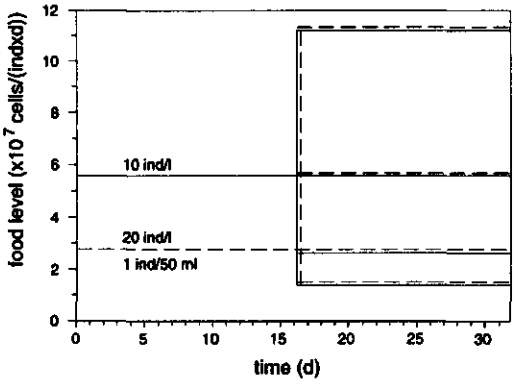

Fig. 1.
After a period of 16 days at constant daily food rations (immediately after the release of the third brood) the parental groups of daphnids were each split up in four subgroups, resulting in different degrees of increased or decreased *C. pyrenoidosa* rations. Parental densities were 10 ind/l in experiment A (solid lines) and 20 ind/l in experiment B or 1 ind/50 ml in experiment C (dashed lines).

Toxicity tests

Just before the seventh brood was released from the brood pouch parents were transferred to the food level in which the acute toxicity tests were to be carried out. Neonates of the seventh brood (<24 h) were exposed to cadmium (as $CdCl_2$) and chromium (as $K_2Cr_2O_7$) during 48 h. The tests were carried out according to Dutch standard procedures (NEN 6501, 1980), with an initial addition of 1×10^8 *C. pyrenoidosa* cells/l. The test medium used was reconstituted water with a hardness of 250 mg/l (as $CaCO_3$) and a pH of 8.3 ± 0.2, and was prepared according to Alabaster & Abram (1965). The tests were carried out in duplicate.

Data processing

In order to compare experiments B and C at all ration levels, mean brood sizes and carapace lengths of parents at day 32 were analysed with a three way and a two way analysis of variance respectively (Sokal & Rohlf, 1981). The factors which may influence brood size are brood number, food ration level and experimental set-up. Factors possibly affecting the ultimate carapace length are food ration level and experimental set-up.

The relation between parental food ration level and carapace length of neonates was calculated by linear regression for experiments A and B. The regression lines were compared using analysis of covariance (Snedecor & Cochran, 1980).

The association between neonate length and lipid index was evaluated by calculating the product moment correlation coefficient (Sokal & Rohlf, 1981).

LC_{50} values were determined by a statistical method according to Kooijman (1981). In case no partial mortality occurred, the 99% confidence limits were set to the highest concentration in which no mortality was observed and the lowest concentration in which all test organisms died (Stephan, 1977).

Results

Feeding studies

The influence of different food regimes on body growth is presented in Fig. 2. Apparently growth is to a large extent affected by food ration level. Furthermore, body growth was also influenced by parental density, as the daphnids of the high density group (20 females/l) remained smaller than those in the lower density (10 females/l). Lowering the food ration levels terminated body growth in both densities, whereas daphnids continued growing at constant and increased ration levels during the second half of the experiment. At day 32 daphnids in the high parental density still lagged behind, being significantly smaller ($P<0.01$) than the individuals in the low density at similar food ration levels.

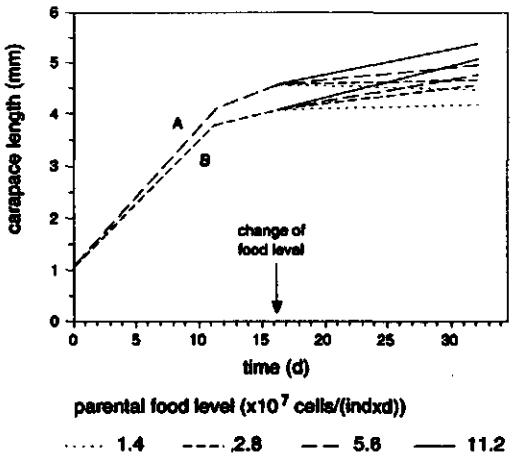

Fig. 2.
The body growth of the parental generation in the low density (A) was stronger than in the high density group (B). After the change of food levels realized on day 16, growth continued at constant and increased food levels, but stopped at decreased daily food rations.

Figs. 3 (experiment A) and 4 (experiment B) demonstrate that the food ration level also regulates brood size and the size of newborn young. Neonates were very small in the first and second brood. Because this effect may be connected with the age of the reproducing female rather than with the food level, broods 1 and 2 were not included in further data analyses. The numbers of young in broods 1 to 3 were higher in experiment A than in experiment B, due to a higher parental density in the latter. The change of ration levels at day 16 did not affect the size of brood 4 in any experiment, because the eggs already had been deposited in the brood pouch. Adaptation to the new environment was not yet clear in brood 5, whereas it was obvious in broods 6 and 7, demonstrating a positive correlation between food level and brood size (Figs. 3 and 4). Despite differences in maternal body size (Fig. 2), brood sizes at ration levels 1.4×10^7, 2.8×10^7 and 5.6×10^7 algal cells/-

(ind×d) were similar at both *Daphnia* densities. However, at the highest food level, females in the low parental density produced substantially larger broods than daphnids in the high density.

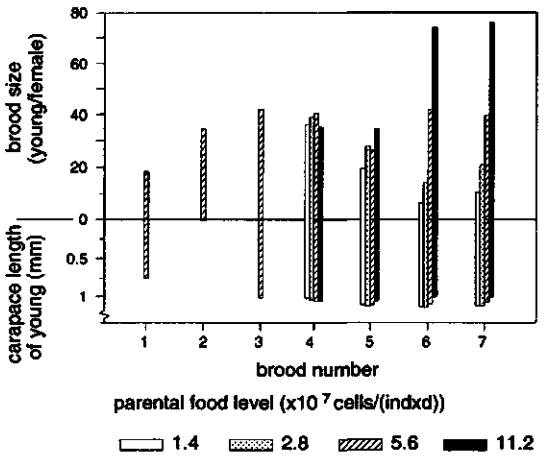

Fig. 3.
The brood sizes and the carapace lengths of newborn *D. magna* in experiment A (parental density = 10 ind/l) adapted to the change in daily food rations within two broods. After an intermediate brood (no. 5), a new stable situation was attained in broods 6 and 7.

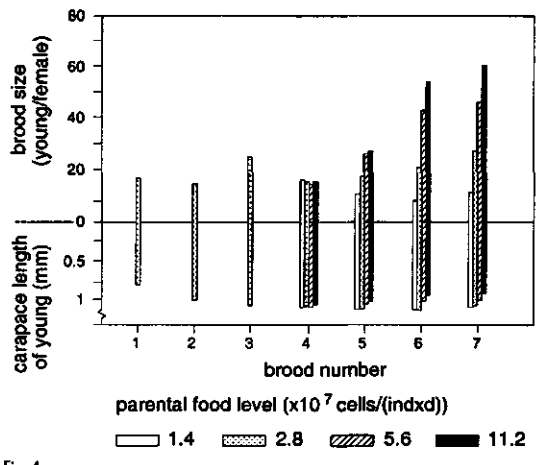

Fig. 4.
The brood sizes and the carapace lengths of newborn *D. magna* in experiment B (parental density = 20 ind/l) showed a similar rate of adaptation to the changes in food ration as in experiment A. Only at the highest food level the number of neonates in broods 6 and 7 lagged behind compared to experiment A.

Figs. 3 and 4 also show that large broods generally consist of smaller neonates than small broods. Because brood size depends on food level, a negative relation between maternal food ration level and neonate body length can be shown (Fig. 5). The slopes of the regression lines calculated for experiments A and B were significantly negative ($P<0.01$). Analysis of covariance revealed a significant difference between the regression coefficients ($P<0.01$), neonate lengths diverging at the highest food levels.

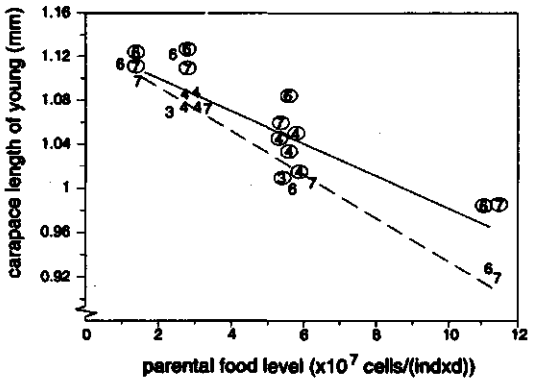

Fig. 5.
Effect of parental food level on neonate carapace length in the low parental density of experiment A (solid line, encircled brood numbers) and in the high density of experiment B (dashed line, open brood numbers). Some broods were omitted from regression analysis, in order to exclude the effects of young age (broods 1 and 2) and incomplete adaptation to the new food level (brood 5). Regression equations are:
$Y = -0.0148X + 1.13$ (solid line) and $Y = -0.0195X + 1.13$ (dashed line), where
X=parental food ration level ($\cdot 10^7$ cells/(ind·d)) and Y=carapace length of neonates (mm). Both coefficients are significantly negative ($P<0.01$).

The body length of a neonate may be regarded as a measurement of the amount of energy transferred from a mother to her young. Furthermore, maternal energy can appear as orange coloured lipid droplets in the transparent body of the neonate. A significant positive correlation ($r=0.67$, $n=34$, $P<0.01$) was found between carapace length and visual lipid index of neonates.

In Fig. 6 brood sizes in experiment C are presented. In this experiment daily food rations as well as the volume of water per animal were exactly the same as in experiment B. A remarkable similarity was found with the brood sizes in experiment B (cf. Fig. 4): broods produced by individually reared daphnids were of the same size as broods produced by grouped daphnids ($\alpha=0.05$). The same holds true comparing carapace lengths at the end of experiments B and C.

During the 32 days of the experiment total mortality was 9% among the parental generation. Some individuals suffered from a deadly obstruction of the gastro-intestinal tract. The cause of this disease, which occurred at all food ration levels, was not clear.

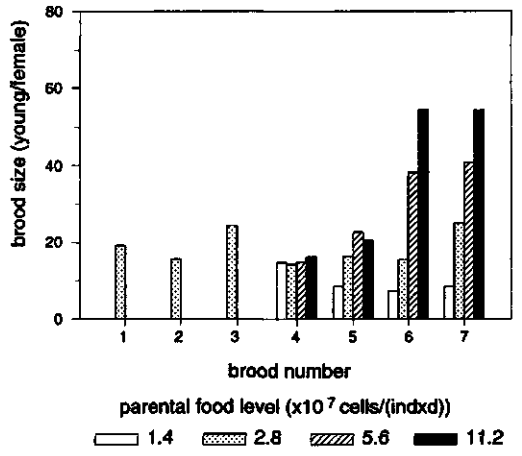

Fig. 6.
Brood sizes in experiment C (parental density = 1 ind/50 ml). Although the pelagic food density in 50 ml vessels was lower than in 1 l vessels, due to rapid algal sedimentation, no difference was observed between the outcome of the test with *D. magna* reared individually, compared to groups of 20 individuals (experiment B).

Sensitivity to heavy metals

The results of the acute toxicity trials with neonates of the seventh brood are given in Figs. 7 and 8. A marked relation appears between the carapace length of a neonate and its sensitivity to cadmium exposure (Fig. 7). The LC_{50} (and 95% confidence limits) ranged from 98 (86-110) µg Cd/l for the smallest neonates to 294 (248-350) µg Cd/l for the largest young. No such effect was found in the acute toxicity tests with chromium (Fig. 8). In all tests the 48-h LC_{50} (and 99% confidence limits) was 1.3 (1.0-1.8) mg Cr/l. Values are based on nominal concentrations.

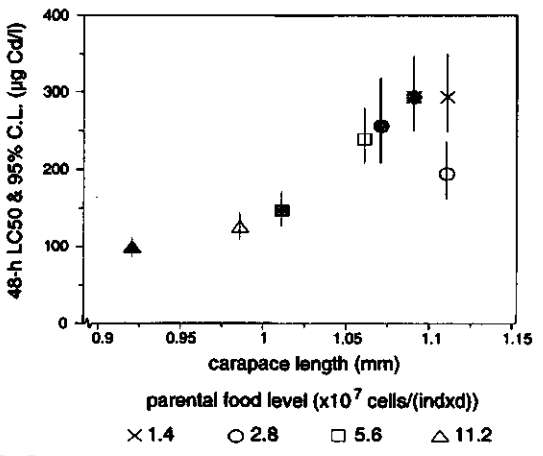

Fig. 7.
Sensitivity of progeny in acute toxicity tests with cadmium. Small neonates from high maternal food levels were three times more sensitive than large young from mothers reared at low food levels. Parental densities were 10 ind/l (open symbols) and 20 ind/l (filled symbols).

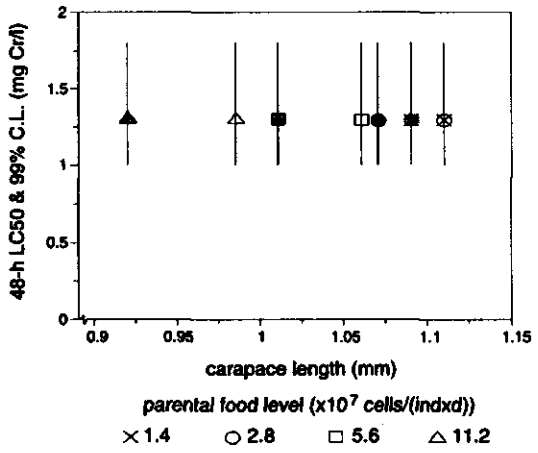

Fig. 8.
Body size of neonates did not influence mortality during acute exposure to chromium(VI). Parental densities were 10 ind/l (open symbols) and 20 ind/l (filled symbols).

Discussion

Reproductive strategy

Our experiments confirm the existence of a reproductive strategy: *D. magna* produces large broods consisting of small young when food is abundant and few but large and fat young are born if food is sparse (Cowgill *et al.*, 1985; Smith, 1963). Adaptation to new food levels takes place in eight days at 20 °C, both to higher and lower food levels. This indicates the possibility of a rapid adaptation to fluctuating food environments in the field.

The results of this study disagree with the publication of Tessier *et al.* (1983), who found a *positive* relation between maternal food level and triacylglycerol transferred to each egg. In our experiments however, both carapace length and visual lipid content of neonates indicate an *inverse* relation between maternal nutrition and energy investment per young. Bradley (pers. comm.) confirmed these results with her observations of increasing egg sizes and decreasing clutch sizes during episodic starvation of *D. magna*.

As early as in 1914, Agar stated, based on a study in a monoclonal population of *Simocephalus expinosus* (cited by Green, 1956): '...when the size of the parent is constant, the size of the eggs, as estimated by the size of the young developing from them, varies inversely as their number. Given the number of eggs to be the same, their size varies as the size of the animal which laid them'. This was true for individuals of the same age. The findings of this study confirm the positive correlation between maternal body size and egg (or neonate) size at a certain brood size (Fig. 5). Adult carapace lengths at brood 7 in experiment B were at all food levels lower than the size of individuals of the same age in experiment A (cf. Fig. 2). Except for the highest food level, the size of brood 7 was similar (cf. Figs. 3 and 4), but the mean neonate length varied as the size of the

mother. This phenomenon may explain the difference between the regression coefficients of the lines depicted in Fig. 5.

Active grazing

Both the volume of medium and the number of algal cells available to each female were identical in experiments B (20 ind/l) and C (1 ind/50 ml). However, the spatial distribution of *C. pyrenoidosa* was not the same, due to a more rapid sedimentation of these algae in small vessels (C) than in large vessels (B). At all food regimes, brood sizes and body growth were the same in both experimental set-ups. This indicates that *D. magna* will actively search for food, and both suspended and sedimented algae are therefore available to them. In experiments with *D. magna* kept with 5 individuals in volumes ranging from 10 to 100 ml, brood sizes and body lengths were related to the number of *C. pyrenoidosa* cells/(ind×d), but not to the volume of test medium (Kooijman, unpublished data). However, it is noteworthy that extremely small or large test volumes may affect the filtering success of daphnids.

Maternal nutrition affects the results of routine toxicity tests

Perhaps the most remarkable result of our experiments is that the sensitivity of young *D. magna* to cadmium depends to a large extent on maternal nutrition. Environmental effects on the sensitivity of *D. carinata* to this metal, as the amount of food during exposure, has been described by Chandini (1989). In addition to this environmental variability experienced after birth, a maternal effect on sensitivity is present. The latter has been taken into account in the generally accepted practice of omitting the first broods for the use in toxicity tests, because they consist of underweight neonates (Cowgill, 1987). Moreover, the present experiments clearly demonstrate the importance of maternal nutrition with respect to neonate sensitivity.

No differences in sensitivity to chromium were observed in our tests, hereby confirming the results of Stephenson & Watts (1984), who also did not find any effects of maternal nutrition when they performed acute toxicity tests with chromium.

In the case of acute exposure to cadmium however, small neonates of well fed mothers were three times more sensitive than large neonates from low maternal nutrition. A similar effect was found by Baird *et al.* who performed acute toxicity tests with 3,4-dichloroaniline (1989). This implies that small neonates not only are a risk group for cadmium, but for other chemicals as well.

The size-dependent response to cadmium, which is absent in acute tests with chromium, could be explained by the presence of a detoxification mechanism in *D. magna* (Bodar *et al.*, 1988). Metallothionein-like proteins are synthesized in the presence of cadmium. Because protein synthesis, or any other detoxification mechanism, will cost energy, large daphnids with high energy reserves may be in a better position than small neonates with a low lipid content. The advantage of a large body is also clear if the toxic action of a chemical merely depends on its uptake and distribution.

The results of the present study illustrate the importance of standardization of food ration level in *D. magna* cultures. Part of the inter- and intra-laboratory variation recognized in ring tests can be explained by the lack of uniformity with respect to culture techniques.

Acknowledgements

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chapter 4

Reproductive strategy of *Daphnia magna*: implications for chronic toxicity tests*

Abstract

Food levels in *Daphnia magna* cultures did apparently not affect the sensitivity of the young in chronic toxicity tests. Variation in test results was mainly due to non-simultaneous replication of the tests.

Reproduction of *D. magna* obviously is related to food rations. At low levels small broods of large neonates were produced, whereas at high food levels many tiny young were born. The ability to withstand starvation was shown to increase with the size of the neonate and with its lipid content. In chronic toxicity tests with cadmium and chromium, however, no unequivocal relationship was found between maternal food level and the sensitivity of the young. Differences in growth and reproduction between small and large neonates were not confirmed in a second experiment. Environmental circumstances during chronic tests were more important than maternal nutrition.

Introduction

This study investigates the importance of the nutrition of *Daphnia magna* cultures with respect to the results of chronic toxicity tests.

A number of studies has shown that some Cladocerans demonstrate a reproductive strategy in response to (changes in) food availability. As early as 1914, Agar found an inverse relation between brood size and body size of neonates in *Simocephalus expinosus*. Later, this phenomenon was also recognized in *D. magna*: females produce many small neonates under favourable conditions, while only few heavy, stress-resistant young are born when food is sparse (Smith, 1963; Cowgill *et al.*, 1985; Enserink *et al.*, 1990). Maternal nutrition therefore, may play an important role in the resistance of the progeny to toxic stress.

Survival of neonates in acute toxicity tests can significantly be affected by maternal nutrition. Both quantity (Baird *et al.*, 1989a,b; Enserink *et al.*, 1990) and quality (Belanger *et al.*, 1989) of the food are relevant. Yet no information is available on effects of maternal nutrition on the performance of the progeny in chronic toxicity tests. Additional variation in test results is generated by the process of non-simultaneous replication. This source of variation was quantified by Gersich

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et al. (1986) for acute tests. In chronic toxicity tests the problem of replication is even more serious (Cabridenc, 1986). The present study focuses on two environmental sources of variation, i.e. maternal nutrition and non-simultaneous replication.

Maternal nutrition may also influence the ability of neonates to withstand starvation. Body size and energy reserves are essential factors when food is scarce. Stored lipids enable Cladocera to prolonge the survival time (Tessier *et al.*, 1983). Goulden & Henry (1984) emphasized the importance of maternal energy reserves for survival of neonates at low food levels. The consequences of maternal investment for fitness of progeny were further investigated by Tessier & Consolatti (1989). They found a positive relationship between neonate mass and the ability to withstand starvation in *D. parvula* and *D. pulicaria*. This relationship appeared to be species dependent: for the small neonates of *D. parvula* the gain in starvation time with increasing size was more pronounced than for the heavier neonates of *D. pulicaria*. Starvation experiments with *D. magna* neonates were carried out by Stephenson & Watts (1984). They raised females on different algal and synthetic diets and measured the quality of the progeny. No effect was found on neonate survival time. These observations led to the question whether the reproductive strategy mentioned above can be regarded as an adaptive trait with regard to the fitness of newborn *D. magna* under starvation conditions.

The objectives of the present study were two-fold. Firstly, the importance of maternal nutrition and non-simultaneous replication in chronic toxicity tests was investigated, using progeny from *D. magna* reared at different food levels. Cadmium and chromium(VI) were taken as model compounds. Secondly, the effect of maternal nutrition on neonate fitness under starvation conditions was studied.

Materials and methods

Experimental conditions

Experiments with *D. magna* were carried out in a constant-temperature room of 20 ± 1 °C with a 12 h photoperiod. The test medium used was 25 µm filtered, UV-treated Lake IJssel water with a pH of 8.1 and a hardness of approximately 225 mg/l (as $CaCO_3$). Daphnids were fed batch-cultured *Chlorella pyrenoidosa*. In order to obtain a stable food quality, the algae were harvested in the exponential growth phase and stored in a dark room at 5 °C for a maximum period of 4 days.

Feeding studies

Daphnids were held in glass aquaria containing 60 individuals in 5 l medium until their 4th brood was born. Twice a day 2.5×10^7 *C. pyrenoidosa* cells/ind were added. The medium was refreshed on Monday, Wednesday and Friday. After 18 days 160 animals were randomly distributed among glass vessels containing 1 l medium. Daphnia density was 10 ind/l. Three different food ration levels were established: 1.4×10^7 (low), 5.6×10^7 (medium) or 11.2×10^7 algal cells/(ind×d) (high). Because daphnids at low food levels produce only few neonates, more animals (120) were kept at low

rations than at medium and high rations (20 individuals at each ration). The medium was refreshed daily. Each day newborn daphnids were counted and discarded. From broods 5, 6 and 7 ca. 50 neonates (<4h) were sampled from each treatment. Carapace length and lipid index (Tessier & Goulden, 1982) were measured microscopically. The experiment was terminated after 32 days.

Chronic toxicity tests with metals (M)

Mothers of test animals were treated as described under 'feeding studies'. Before their 7th brood was born gravid females were transferred to the food level which was used in the chronic toxicity tests. Neonates of this brood (<24h) were exposed to cadmium (as $CdCl_2$) or chromium (as $K_2Cr_2O_7$) in semi-static 21-day experiments. They were randomly distributed into groups of 10 animals each, among 11 cadmium concentrations, 8 chromium concentrations and a control (test M1). The experiments were performed in duplicate. The concentration ratio was 1.8. Daphnids were fed 3×10^7 algal cells/(ind×d). The number of surviving females and the number of neonates produced were recorded daily. Newborn young were discarded from the test vessels. Test vessels contained 500 ml medium, which was renewed on Monday, Wednesday and Friday. In case of mortality the volume of test medium was reduced with 50 ml for each dead female. After 3 weeks the experiments were terminated. Carapace lengths of surviving adults were determined with a microscope. The experiment with cadmium was repeated (test M2).

Starvation experiment (S)

The maternal generation was held in large aquaria, containing 60 females in 5 l medium. The medium was changed three times a week and the animals were fed 2.5×10^7 algal cells/ind twice a day. After the release of their 7th brood, 10 females were transferred to each of three food regimes, i.e. 1.4×10^7, 5.6×10^7 or 11.2×10^7 algal cells/(ind×d). The females and their young were treated as described under 'feeding studies'. Just before the 9th brood was released from the brood pouch, gravid females were transferred to 0.45 µm filtered standard water, prepared according to Alabaster & Abram (1965). The hardness of this medium was 250 mg/l (as $CaCO_3$) and the pH was 8.3 ± 0.2. The water was thoroughly aerated prior to filtration. Juveniles were held individually in small glass beakers containing 50 ml of medium. Daily the animals were transferred to freshly filtered medium. Moults and survival time were recorded. The starvation experiment (test S) was carried out in tenfold.

Chemical analyses

Total acids exchangeable metal concentrations in the toxicity experiments were measured by Atomic Absorption Spectrometry. The results of the experiments are given in actual concentrations.

Data processing

LC_{50} values were determined by a statistical method according to Kooijman (1981). In case no partial mortality occurred 99% confidence limits were set to the highest concentration in which no

mortality was observed and the lowest concentration in which all test organisms died (Stephan, 1977).

The intrinsic rate of natural increase (r_m) for each replication in the life table experiments was calculated by substitution of the daily observed effects on survival and reproduction in the formula of Lotka (1913):

$$\sum_{x=0}^{\infty} l_x m_x e^{-r_m x} = 1 \qquad (1)$$

where l_x is the proportion of the individuals surviving to age x (days) and m_x is the number of juveniles per surviving female between age x and $x+1$. The total number of young per female produced in 21 days (cumulative m_x) was also calculated.

Differences in the r_m, cumulative m_x and carapace length between the controls and test concentrations were tested using Dunnett's method (Dunnett, 1955, 1964; $P<0.01$). The lowest concentration causing a statistically and biologically significant decrease with respect to the control is denoted as the LOEC (Lowest Observed Effect Concentration). Analogous to a guideline for prolonged toxicity studies with *D. magna* (EC, 1986) a LOEC is considered biologically relevant if the difference with respect to the control exceeds a fixed percentage. These percentages were based on the within-test coefficients of variation of control parameters in a series of experiments carried out between 1983 and 1989 in our laboratory. They were calculated according to the formula:

$$D = \frac{4\overline{C.V.}}{\sqrt{n}} \qquad (2)$$

where D is the minimum difference with respect to the control (%), $C.V.$ is the mean historical coefficient of variation (%) and n is the number of replicates within a test. A factor of 4 was applied to secure a 99.99% confidence interval. For the total number of young per female, the r_m and the carapace length the minimum differences considered relevant were 13, 7 and 3%, respectively.

The effects of maternal food ration and non-simultaneous replication on the results of the chronic toxicity tests were examined by a two-way analysis of variance (Sokal & Rohlf, 1981). Parameters considered were the 21-d LC_{50}, as well as reproduction and growth in the controls. For multiple comparisons of means that were not ordered along a toxicant gradient the Student-Newman-Keuls test (Sokal & Rohlf, 1981) was used.

In the starvation experiment with neonates the effect of maternal ration on the number of moults recorded before death was tested with Fisher's exact test in a 2×2 table (Sokal & Rohlf, 1981).

Results

Feeding studies

Reproductive adaptation of the parental generation to the new food condition occurred within three instars. Brood size was positively correlated with food level, whereas neonate size decreased with increasing rations (Table 1). Between food levels the neonate carapace length differed significantly ($P<0.05$). Large neonates carried relatively large energy reserves. The results were consistent in all experiments.

Table 1.

Brood sizes and neonate body lengths at three different food levels: $1.4 \cdot 10^7$ (low), $5.6 \cdot 10^7$ (medium) and $11.2 \cdot 10^7$ (high) cells/(ind·d). These neonates were used in chronic toxicity tests with cadmium and chromium(VI) (tests M1 and M2) and in a starvation experiment (test S).

test	maternal food level	brood size	neonate body length ± sd (mm)	neonate lipid index
M1	low	10	1.10 ± 0.03^a	3
	medium	25	1.05 ± 0.03^b	1
	high	57	0.97 ± 0.03^c	0-1
M2	low	14	1.08 ± 0.03^a	2
	medium	51	0.98 ± 0.04^b	1
	high	66	0.91 ± 0.06^c	1
S	low	7	1.10 ± 0.02^a	3
	medium	31	1.07 ± 0.03^b	1-2
	high	57	0.97 ± 0.03^c	1

Within an experiment, values followed by the same letter do not differ significantly ($P<0.05$).

Chronic toxicity tests

A comparison of the performance of individuals which were not exposed to toxicants is given in Table 2. The food condition of the mothers of the test animals slightly affected the total number of young produced during the test (M1 and M2), the intrinsic rate of natural increase, r_m (M1) and the carapace length attained in 21 days (M2). However, the relationship between maternal investment and performance of the progeny was reversed in replicate tests. In experiment M1 reproduction of the small, lean progeny from high food mothers was enhanced in comparison with both other groups. In contrast, the results of experiment M2 showed a more vigorous reproduction and a larger adult body size of progeny from the lowest maternal ration, which were large and fat at birth.

In this experiment no significant difference was found between young from the intermediate and high maternal ration.

Table 2.
Effects of maternal nutrition and non-simultaneous replication on reproduction and body growth of *Daphnia magna* in 21-d control tests. The test animals were produced by mothers reared at different food levels.

test	maternal food level	total young per female	r_m	carapace length (mm)
M1	low	53.6[a]	0.370[a]	3.98[a]
	medium	56.7[a]	0.362[a]	3.95[a]
	high	67.4[b]	0.397[b]	3.96[a]
	mean	59.2	0.376	3.96
M2	low	75.0[b]	0.360[a]	4.16[b]
	medium	64.9[a]	0.351[a]	4.07[a]
	high	60.8[a]	0.330[a]	4.06[a]
	mean	66.9	0.347	4.10

The length of the juvenile period was not affected by maternal food level, but there was a difference between tests. In tests M1 and M2 the first brood was born on day 8 and 9 respectively. This is reflected in the value of the r_m which is relatively high in test M1 compared to test M2. Analysis of variance considering reproduction and growth in controls showed that non-simultaneous replication was the main source of variance.

In the 21-d toxicity experiment with chromium(VI) a trend in the sensitivity of progeny from different maternal nutrition was observed (Table 3). Young from the lowest maternal food level were slightly less sensitive than those from higher food levels. This appeared in the LC_{50} (not significant), and the LOEC's for the cumulative m_x and the r_m. Comparable results were found in the first experiment with cadmium (Table 3). Differences were very small, but the trend was consistent. In the second experiment with cadmium however, the trend was not consistent anymore. The LC_{50} for cadmium was significantly affected by non-simultaneous replication, but not by the feeding condition of the mothers of the test animals, as was demonstrated by analysis of variance. An example of the effects of both factors on the dose-response relationship for the rate of increase (r_m) is given in Fig. 1. In the first test with cadmium (Fig. 1, M1) the r_m of progeny from low maternal nutrition was relatively low and slightly less sensitive to cadmium than the r_m of both other groups. In the second test (Fig. 1, M2) however, differences between groups were smaller and effect concentrations were higher than in the first test. In all groups dead neonates and aborted eggs were found at concentrations higher than 2 µg Cd/l.

Table 3.

Concentrations of chromium(VI) and cadmium causing adverse effects in 21-d chronic toxicity tests. The exposed animals were produced by mothers held at different food levels.

					LOEC		
substance	test	maternal food level	LC_{50}	(95% C.L.)	total young per female	r_m	carapace length
Cr(VI)	M1	low	0.44	(0.38-0.51)	0.94	0.94	0.16
(mg/l)		medium	0.38	(0.30-0.50)	0.53	0.53	0.27
		high	0.38	(0.32-0.56)	0.27	0.53	0.09
Cd	M1	low	4.9	(4.1-6.0)	4.4	4.4	4.4
(µg/l)		medium	3.3	(2.9-3.8)	4.4	4.4	2.3
		high	3.5	(2.8-4.3)	2.3	2.3	2.3
	M2	Low	8.3	(7.7-9.1)	2.1	7.0	4.0
		medium	7.4	(6.6-8.3)	4.0	7.0	4.0
		high	7.4	(6.3-8.8)	4.0	7.0	7.0

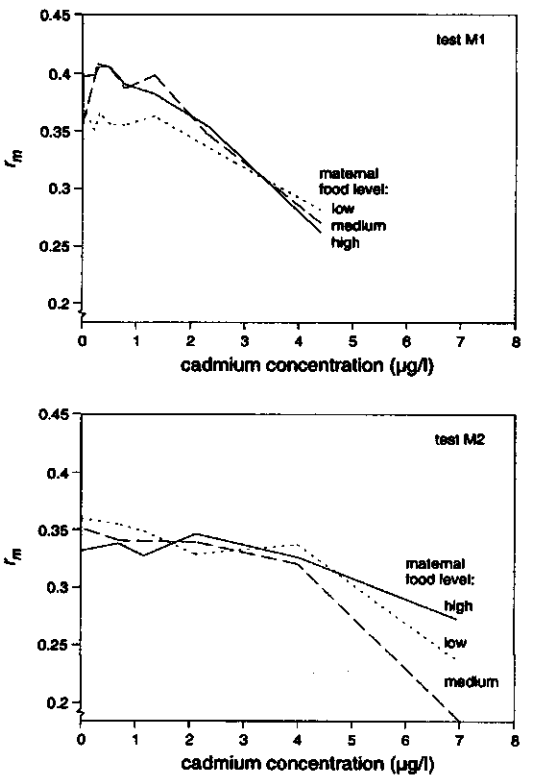

Fig.1.

Effect of cadmium on the per capita rate of increase (r_m) in chronic tests. The test animals were produced at different maternal food rations.

A high percentage (26%) of males was recorded among the progeny of the lowest maternal ration in test M2. They were detected and removed on the sixth day of the experiment. Neither mortality nor reproduction was observed by that time. Males were not included in the data analyses.

Starvation experiment

The median survival time of starving neonates was 6-7 days. It was not affected by maternal food level (Fig. 2). However, an effect on the shape of the survival curves was found. The curve for neonates from the lowest maternal ration was steeper than the curves of both other groups, indicating less individual variation and a somewhat longer survival time for the majority of young in the former treatment. Although they did not survive much longer, the large-bodied, fat young of mothers held at low nutrition spent more energy on growth than small young from the higher maternal food levels. This is reflected in the number of moults recorded (Fig. 3). Most young produced at the lowest ration died in their 3rd juvenile instar, whereas neonates from the intermediate and high rations only reached the second instar. This pattern was statistically significant ($P<0.01$).

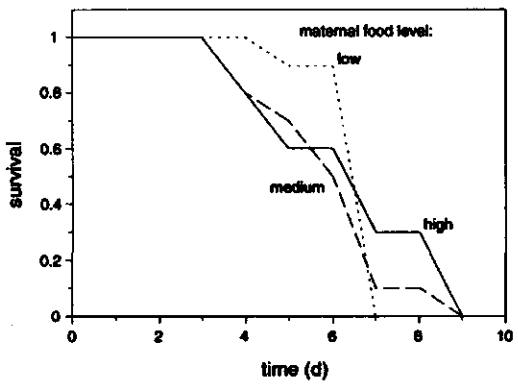

Fig.2.
Survival of neonates at starvation. These young were born from females held at different food rations.

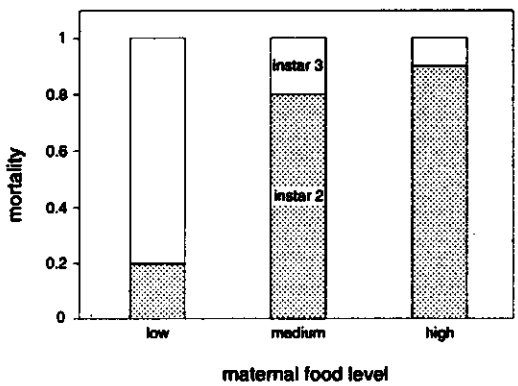

Fig. 3.
Development of neonates at starvation. The frequency of deaths occurring in the second and third juvenile instar is indicated. These young were produced at different maternal food rations.

Discussion

In a previous experiment it was shown that maternal nutrition of *D. magna* significantly affected the sensitivity of the progeny in acute 48-h toxicity tests. Large broods consisting of small neonates were produced at abundant food and few but large and fat neonates were born at low food levels. The small young were three times more sensitive to cadmium than the large ones. No differences were found in tests with chromium(VI) (Enserink *et al.*, 1990). Consistent results were found in several tests carried out non-simultaneously. It was clear that variation due to maternal factors may by far exceed the normal intra-laboratory variability. For instance, Gersich *et al.* (1986) demonstrated that the coefficient of variation for non-simultaneous replication of acute tests is about 4%.

The present study shows that in chronic toxicity tests with *D. magna* the results were different. In the first experiment (M1) with chromium(VI) and cadmium a similar trend was observed as in the acute tests with cadmium, though much less pronounced. However, in the second trial with cadmium (M2) the large neonates originating from the low food level appeared to be slightly more sensitive. This automatically leads to the conclusion that the maternal food level is a much less important source of variation in chronic than in acute tests.

A comparison of growth in the control groups of the present study showed that large neonates maintained their lead over smaller young during 21 days, indicating that the condition at the moment of birth can result in a lasting advantage. No trend was found in the number of young per female and the per capita rate of increase r_m. In contrast, Tessier & Consolatti (1989) found an increase of the body weight of primiparae as well as the size of the first clutch with body weight at birth for *D. pulicaria*. However, there was no further gain in performance in the higher neonate size classes. Moreover, differences in growth and reproduction disappeared when food conditions improved in *D. parvula*. From the data of Tessier & Consolatti it can be concluded that food conditions are of paramount importance for the evaluation of the consequences of size at birth. At low food levels differences in neonate fitness are evident, especially in the lower range of size classes, whereas differences can be obscured at high food levels. In our experiments, no clear relationship between neonate size and the number of progeny was observed. This can be explained by the relatively high food level in our toxicity tests.

Postnatal conditions have played an important role in the present study. From the observed differences in reproduction and growth in control tests carried out non-simultaneously it can be concluded that conditions varied between tests. The quality of Lake IJssel water, which was used as a medium, and of the algal food may fluctuate. Maternal factors, i.e. the food conditions of *D. magna* producing the test animals, appeared to be a less important source of variation. From a comparison with acute studies reported earlier (Enserink *et al.*, 1990), it can be concluded that maternal factors become less important with time, while the influence of postnatal conditions increases.

In our experiments size at birth did not affect the median survival time upon starvation. Neonates produced at three maternal food levels were used, with mean carapace lengths ranging

from 0.97 mm (high food level) to 1.10 mm (low food level). The overall coefficient of variation (C.V.) for neonate length was 5.6% for pooled observations. In the experiments of Tessier & Consolatti (1989) one maternal food level was applied. The C.V. for neonate length was 4.6% in several clones of *D. parvula* and *D. pulicaria*. The ability to withstand starvation increased with neonate size, but the increase was stronger in the small species *D. parvula* than in the larger *D. pulicaria*. Despite a similar C.V. in our experiments, this effect was not observed. Probably, the trend observed by Tessier & Consolatti, i.e. a decreasing gain in survival time with neonate body size, can be extrapolated up to the large neonates of *D. magna*. This idea seems to be endorsed by the results of Stephenson & Watts (1984), who also failed to find an effect of maternal nutrition on starving neonates of *D. magna*. Unfortunately, data on the body size of neonates were not provided. Similar studies of the effect of maternal food quality on neonate size in this species showed that large broods generally consisted of lighter neonates than those produced in smaller broods (Cowgill *et al.*, 1985). In this study clutch sizes ranged from 10 to 33. Since this range was also produced at different diets in Stephenson & Watts' studies, we can assume the existence of significant differences in the body size of their neonates. Thus, these observations also appear to agree with the hypothesis that large *Daphnia* neonates do not extend their survival time during starvation. The question arises whether or not an increase of maternal investment per neonate at decreasing resource levels can be regarded as an adaptive trait in the large species *D. magna*. If so, some other advantage of large neonate size must exist.

A remarkable result of the present study was that large, fat neonates produced at the lowest maternal food level accomplished a more rapid development during starvation than the smaller neonates. In other experiments carried out in our laboratory provisioning of the ovaries started during the third juvenile instar, i.e. these organs became capable of reproduction. Thus, at low food maternal investment is sufficient to reach the adolescent instar without any additional food, whereas at intermediate and high food levels extra energy is needed. Apparently, the large young gave priority to growth and development at the expense of survival. In a variable environment early maturation will be advantageous, because it enables an individual to reproduce rapidly when resource levels increase. If low food conditions continue a large body is beneficial, according to the size-efficiency theory of Brooks & Dodson (1965). It is assumed that filtration efficiency increases and weight-specific metabolic rate decreases with body size. Hence, the production of large neonates at low food levels might be adaptive at stable low food conditions as well as for fluctuating resource levels.

Although *D. magna* is widely used as a test organism in standard ecotoxicity studies, guidelines for the performance of such tests hardly consider important sources of variation. For instance, factors like feeding conditions and the quality of the medium are not yet standardized in the international protocols (e.g. EC, 1986). These factors have been recognized as potential sources of variation (Buikema *et al.*, 1980; Stephenson & Watts, 1984; Chandini, 1988, 1989; Baird *et al.*, 1989a; Persoone *et al.*, 1989). In addition, clonal variation can affect the results of chronic toxicity tests (Soares *et al.*, 1992).

From the results of the present chronic studies and our former acute studies we conclude that the nutrition of *D. magna* cultures deserves more attention in test protocols. Adequate standardisation of food levels requires inter-laboratory comparisons and frequent verification. It yields increased homogeneity of test organisms and, thereby, improved reproducibility of test results.

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chapter 5

Influence of food quantity and lead exposure on maturation in *Daphnia magna*; evidence for a trade-off mechanism*

Abstract

1. The start of reproduction is an important life-history trait in both ecology and ecotoxicology. Effects of food availability and toxic stress on maturation in female *Daphnia magna* are presented.

2. Five instars to maturation were observed under conditions of moderate to high food levels. Age and size at subsequent stages of the maturation process, i.e. ovary provisioning and egg deposition, depended on the rate of body growth.

3. At low food, a trade-off between reproduction and growth occurred. Within the same environment, some females reproduced at early age and small size, whereas others gave priority to growth over reproduction. In the latter resorption of yolk from the ovaries was observed.

4. Except for the production of malformed young, effects of lead on maturation resembled increased food stress. At abundant food toxic effects were different to effects at low food. The results of standardized laboratory tests may therefore underestimate toxic effects at reduced food availability.

5. The phenomena observed in our studies can only partly be explained by a food independent size threshold for maturation, which initiates resource allocation to reproduction; a minimum food uptake is required to enable the production of eggs.

Introduction

In ecotoxicology, crustaceans of the genus *Daphnia* are frequently used as test animals. Although these tests are valuable in themselves, the implications of toxic effects on the individual for higher levels of organisation (population and community) are important. Life-history studies can clarify the mechanisms that determine the reaction of an individual to its environment, including toxic stress. Moreover, these mechanisms make up the basis of population dynamics.

Maturation is one of the major life-history traits. Within a genotype these traits are subject to modifications in reaction to environmental conditions. A so-called reaction norm for maturation describes the full set of phenotypes that can be expressed (Stearns, 1992). Although age and size at reproduction are among the most commonly measured life-history traits in *Daphnia*, only a few authors have investigated the maturation process itself.

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According to Zaffagnini (1987) the production of a brood takes three instars in *D. magna* Straus. These instars can be called IM-1 to IM-3 (Bradley *et al.*, 1991). Oogenesis and differentiation of oocytes from nurse cells occur in the first of these instars (IM-1). At this point, lipid droplets appear in the ooplasm. Mass provisioning of oocytes with maternal reserves and yolk formation takes place in the following instar (IM-2). The deposition of eggs into the brood pouch and embryogenesis occur in the third instar (IM-3) called the primiparous instar in the case of first reproduction. The major investment of energy into the eggs occurs during the first half of IM-2 (Bradley *et al.*, 1991), although McCauley *et al.* (1990b) reported a small commitment to reproduction at the end of IM-1, which is visible as a darkening of the ovaries.

For several Cladocera increasing food availability causes increased size and reduced age at first reproduction (e.g. Porter et al., 1983; Tillmann & Lampert, 1984; Taylor, 1985). However, Perrin (1989) found a constant, food-independent size at which the provisioning of ovaries occurs (IM-2) in *Simocephalus vetulus*. Lynch (1989) proposed a critical size for the onset of reproductive investment (IM-2) in *D. pulex*. Although this threshold is the same at all food concentrations, size in the next instar (IM-3) depends on growth rate and thus on food level. A more complicated pattern was found by McCauley *et al.* (1990a) in *D. pulex*. Their studies involved low food levels, representative of field conditions. The smallest primiparae were found at intermediate food levels. Both higher and lower food levels caused individuals to mature at larger sizes. The former was owing to high growth rates and the latter to the inability to accumulate enough energy to produce an egg in the first adult instar. Indeed, delayed reproduction and increased sizes of primiparae are normal phenomena in the field (Lampert, 1988). McCauley *et al.* (1990a) also stressed the existence of a minimum age at maturation, occurring at high food supplies. As food level increases, the developmental rate approaches a maximum.

Ebert (1992) also proposed a food-independent size threshold for maturation of *D. magna*, i.e. between the pre-preadolescent instar (IM-0) and the preadolescent instar (IM-1), in accordance with McCauley *et al.* (1990b). At high food levels the pre-preadolescent instar often coincides with the second juvenile instar. Lower food levels as well as small size at birth increase the number of instars to the threshold and thus to maturity. Ebert (1991) described reaction norms for length or age at maturity, in relation to food level and length at birth, which are essentially continuous within instar groups, but discontinuous between instar groups. By definition, females mature in the same number of juvenile instars within an instar group.

Hence, food availability and body size seem important determinants for maturation. However, there is no consensus about the influence of food on the consecutive stages of maturation. The main objective of this study is to confirm this influence in *D. magna*, with emphasis on the accumulation of yolk in the ovaries, being the first sign of substantial allocation of energy to offspring. Conditions of low food are of prime interest, since they are expected to provoke interesting allocation strategies (cf. McCauley *et al.*, 1990a). Starvation experiments are performed to evaluate the fitness of progeny from the first brood.

Another objective was to investigate the extent to which maturation can be affected by a toxic substance. Time to first reproduction is measured routinely in chronic toxicity tests with *Daphnia*,

but the relevance of toxic effects on maturation has received little attention compared to the number of young produced during the test (e.g. Van Leeuwen *et al.*, 1985). In the present study combined effects of food availability and a toxicant on maturation are evaluated. Lead was chosen as a model substance, because of its known effect on the time to first reproduction in *D. magna* (Enserink *et al.*, 1991).

Materials and methods

Experimental conditions

Experiments with *D. magna* from our laboratory stock were carried out at 20 ± 1 °C with a 12 hour photoperiod. The medium used was a 1:1 mixture of UV-treated Lake IJssel water and Dutch Standard Water (NPR 6503, 1980). This was 0.45 μm filtered. The pH was 8.1 and the hardness 217 mg/l as $CaCO_3$. Daphnids were fed batch-cultured green algae (*Chlorella pyrenoidosa* Chick). To obtain a stable food quality, the algae were harvested in the exponential growth phase and stored in a dark room at 5 °C for a maximum of 4 days. The number of cells in newly prepared medium was checked daily by means of a Coulter Counter (Coulter Electronics; Harpenden, England). The food ration given to test animals is expressed as number of *Chlorella* cells per individual per day: /(ind×d).

Test animals were born from cohorts held at a food ration of *c.* 10^8 *Chlorella* cells/(ind×d). Immediately before the release of the neonates used in the experiments, gravid females were transferred to algae-free medium.

Food experiment

This experiment was carried out with 10th clutch neonates produced by the same maternal cohort. At the start of the experiment daphnids (0-3 h) were randomly distributed among beakers containing 50 ml of medium. Five different rations were used: 7×10^5, 16×10^5, 30×10^5 and 50×10^5 cells/(ind×d) (these represent resource limitation) and 500×10^5 cells/(ind×d) (allows maximum growth, but not nescessarily maximum reproduction). Using the mean carbon content of a *C. pyrenoidosa* cell (6.9×10^{-6} μg C), carbon concentrations of fresh medium can be calculated as 0.097, 0.22, 0.41, 0.69 and 6.9 μg C m/l respectively. At each ration 20 daphnids were held, one per beaker. The water was replaced daily. In order to estimate the length (top of the head to the base of the tail spine) of the daphnids at birth, carapace lengths of 10 individuals of the same cohort were measured under a dissecting microscope.

Test animals were observed until the first brood was released from the brood pouch, or until death. Observations by eye were made at least daily (during weekends), but usually four times a day. Growth, moulting, ovary development and deposition of eggs were recorded. Special efforts were made to follow the accumulation of yolk in the ovaries (Fig. 1).

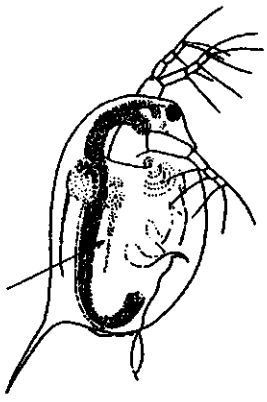

Fig. 1.
Position of ovaries in D. magna, indicated by the arrow. Accumulated yolk, which is visible as a greyish substance, shines through the transparent carapace.

Microscope observations were taken at smaller time intervals (2-4 h) at about the estimated time of ovary provisioning. Carapace length was measured just after each moult, but not before the carapace was hardened. The number of young in the first clutch was counted. In each ration group body length and lipid index (Tessier & Goulden, 1982) of 20 neonates (0-24 h) were determined. These animals were used in the starvation experiments (below). During the experiment five animals were killed accidentally.

Starvation experiment with neonates

Just before the release of their young, mothers were transferred to filtered medium without food. Neonates were held individually in 50 ml of filtered medium. Each day the medium was changed and survival recorded.

Lead experiment

Two food regimes and five concentrations of lead ($PbCl_2$, Merck, 99.9% purity) were applied. Rations were 23×10^5 (low) and 160×10^5 cells/(ind×d) (high). Initial carbon concentrations were 0.32 and 2.2 µg C m/l respectively. The latter food ration is often used in standard toxicity tests. Nominal lead concentrations were 0, 0.10, 0.32, 1.0 and 3.2 mg Pb/l. At the start of the experiment eighth clutch neonates (0-3 h) were distributed among glass vessels containing 50 ml of medium. Ten *D. magna* were kept individually at each treatment. The medium was changed daily. Growth and maturation were recorded as described for the food experiment.

Chemical analyses

Lead concentrations were determined by atomic absorption spectrometry. Both newly prepared (total Pb) and aged (total and dissolved Pb) medium were analysed. Dissolved lead represented

76% of the total lead in the high food ration and 81% in the low ration. The mean decrease of total lead in all concentrations was 16% in 24 h. Test results are given in actual concentrations of total lead, i.e. the arithmetic mean of concentrations in fresh and aged medium.

Data processing

The proportion of total carbon weight investment spent on reproduction during the first instar in which mass provisioning of ovaries occurs (IM-2) was calculated according to McCauley *et al.* (1990a):

$$proportion\ to\ reproduction = R(G+R+M)^{-1} \quad (1)$$

where: R= reproduction, i.e. mean mass of a neonate × brood size (µg C)

G= growth, i.e. body mass of instar IM-3 - mass of instar IM-2 (µg C)

M= maintenance, i.e. mass spent on maintenance during the intermoult period between IM-2 and IM-3 (µg C)

For the conversion of carapace length measurements to carbon mass a regression on data from a previous experiment (our unpublished data) was used. Neonates, juveniles and non-ovigerous adults were included. Dry weights of individual *D. magna* were measured, using an electronic microbalance (Sartorius, type XM 1000P; Göttingen, Germany) with a precision of 1 µg. A linear regression was performed on ln-transformed data (n=141, r^2=0.93). Data from low- and high food cultures were pooled, as no significant difference was found between regression lines for separate cultures (P>0.05). The relationship was transformed to carbon mass assuming C=0.42DW (Lampert,1977):

$$W = 4.87L^{2.45} \quad (2)$$

where: L= carapace length (mm)

W= mass (µg C)

Daily maintenance was calculated according to Nisbet *et al.* (1989) and Gurney *et al.* (1990), with modifications based on the work of Bohrer & Lampert (1988) and Glazier (1991). In *D. magna* carbon loss owing to respiration increases linearly from starvation conditions until the incipient limiting level and attains a maximum value thereafter (Bohrer & Lampert, 1988). Respiration rate is more or less isometric with body mass (Glazier, 1991). Measurements on brooding females yield underestimations for adult respiration rate during most of the incubation period, as eggs in stage 1 to 4 (after Threlkeld, 1979) respire at about 33% of the adult rate. The respiration rates of Bohrer & Lampert (1988), who used brooding females, were corrected for the contribution of eggs, to make them applicable to *Daphnia* of any size. In the present studies, food levels decreased significantly between daily transfers, especially at low rations. In order to compare these feeding conditions with the constant food levels of Bohrer & Lampert (1988), we calculated the mean food

concentration for 24 h, using the grazing equations in Gurney *et al.* (1990). The mean body length in stage IM-2 was used as an estimate of L.

The calculation of maintenance and grazing as described above is based on parameter estimates for both *D. magna* and *D. pulex*. We assume similar physiological processes in these species, as they are closely related.

Results

Effect of food ration on juvenile growth and maturation

The growth rate of juveniles increased with food ration, and decreased with age (Fig. 2). Intermoult periods increased with decreasing ration (cf. Table 2). For instance, at the fifth moult animals from the lowest ration lagged 5 days behind the fast-growing individuals at higher food rations.

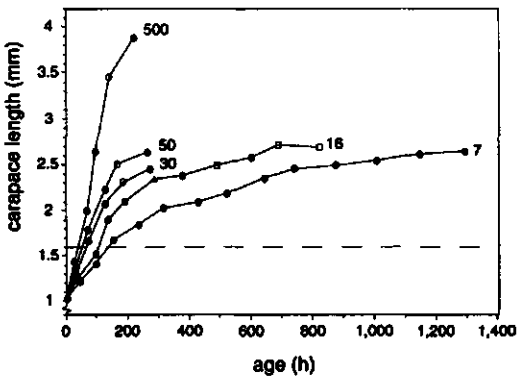

Fig. 2.
Growth and maturation at different food rations. Markers represent mean values of 20 individuals or less, dependent on survival. Open symbols indicate size and age at the beginning of the primiparous instar. Early and late primiparae at $16 \cdot 10^5$ cells/(ind·d) are indicated with a triangle and a square respectively. The broken line describes a hypothetical size threshold for maturation. Food rations are given in 10^5 cells/(ind·d).

An example of individual variation is given in Fig. 3. Variability of body length within instars was similar between food levels (Student-Newman-Keuls test, $P>0.05$). The mean coefficient of variation was 2.6%. Moulting was synchronised in the first instars. Individual variation increased in the course of the experiment, especially at the lower food rations.

Egg deposition occurred in the fifth juvenile instar at rations of 30×10^5 cells/(ind×d) and higher. Body length of primiparous females increased with food level, and their age decreased (Fig. 2). Comparable patterns were found for the adolescent instar (IM-2) and the preadolescent instar (IM-1).

The animals at ration 16×10^5 cells/(ind×d) deserve special attention. Within one cohort, and with a minimum of environmental variation, two maturation groups can be distinguished (Fig. 2).

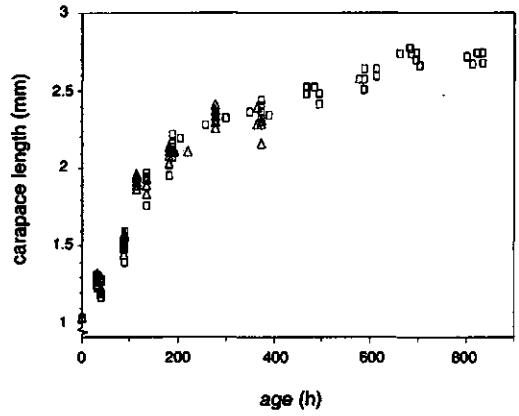

Fig. 3.
Individual variability with respect to growth, at the food ration of $16 \cdot 10^5$ cells/(ind·d). Symbols indicate individual measurements. Females that reproduce at low age and higher age are represented by triangles and squares respectively.

The 'early' females (n=11) laid eggs in the sixth instar, whereas the 'late' (n=7) delayed reproduction until instars 8 to 11. Growth curves of these groups were indistinguishable to the sixth instar (Fig. 3). However, in the seventh instar the mean body size in the early group was significantly lower than in the late group (i.e. mean±SD 2.27±0.06 mm and 2.37±0.05 mm respectively; P<0.01). Moulting times remained similar at this stage.

Most animals at the lowest ration (i.e. 7×10^5 cells/(ind×d)) died without reproducing. Median survival time was 62 d. Only one female gave birth to a neonate; she died shortly afterwards.

A threshold size for maturation (Ebert, 1992) can be located between the second (IM-0, 1.30 ± 0.03 mm) and third (IM-1, 1.69 ± 0.05 mm) instar at 30×10^5 cells/(ind×d), but above 1.52 ± 0.05 mm; i.e. the length of the third instar (IM-0) at ration 16×10^5 cells/(ind×d). The 99% confidence intervals of the last pair of mean lengths meet at 1.60 mm. This critical length is exceeded in the third juvenile instar at food levels $\geq 30\times10^5$ cells/(ind×d) and in the fourth instar at 16×10^5 cells/(ind×d), resulting in the deposition of the first clutch during the fifth and sixth instars respectively (cf. Fig. 2). However, the late group at 16×10^5 cells/(ind×d) as well as the daphnids at 7×10^5 cells/(ind×d) do not respond to this threshold.

The effects of ration on maturation have been summarized in Table 1. Ovary provisioning (instar IM-2) and egg laying (instar IM-3) were significantly delayed at lower food levels. The effect of ration on body length at maturation was not unidirectional. Individuals receiving 30×10^5 cells/(ind×d), as well as the early breeders at 16×10^5 cells/(ind×d) were significantly smaller than the other groups. At higher rations lengths at maturity significantly increased, owing to high growth rates. Large body sizes were also observed among the late breeders at the low ration of 16×10^5 cells/(ind×d). Delay of reproduction and continued growth in this group caused large body sizes at maturation. Essentially the same relationship between food level and body size was found for the instars IM-2 and IM-3, although less pronounced in the former instar.

Table 1.

Effect of food ration on the first clutch. Mean values for mothers and young are given. At the ration of $16 \cdot 10^5$ cells/(ind·d) 'early' and 'late' breeders are distinguished.

		primiparae				progeny			
		IM-2		IM-3					
food ration (10^5 cells /(ind·d))		n	age (h)	body length (mm)	age (h)	body length (mm)	brood size	body length (mm)	lipid index	LT_{50}† (d)
500		20	86[a]	2.63[d]	134[a]	3.45[d]	19[d]	0.77[a]	1	4.0[a]
50		20	113[b]	2.23[b]	159[b]	2.51[b]	2.4[c]	0.93[b]	1	5.0[b]
30		19	115[b]	2.11[a]	183[c]	2.34[a]	1.9[b]	0.94[b]	1	9.0[c]
16	early	11	185[c]	2.09[a]	278[d]	2.33[a]	1.0[a]	0.93[b]	2	8.5[c]
	late	7	573[d]	2.51[c]	674[e]	2.67[c]	1.1[a]	1.02[c]	2	6.0[bc]
7		1	421	2.20	517	2.24	1	0.72*	3	12.5

Values indicated by the same letter do not differ significantly (Student-Newman-Keuls test, $P<0.01$).

†Median survival time. Values were compared with the Kruskal-Wallis test ($P<0.01$).

*Neonate was born prematurely, hence size is underestimated.

Clutch size decreased with food level (Table 1). At the lowest rations only one egg was produced and there was no difference between early and late reproduction at 16×10^5 cells/(ind×d). However, in the early group significantly smaller neonates were produced than in the late group. The smallest neonates were born at 500×10^5 cells/(ind×d). Lipid index of offspring was largest at low food rations.

Coupled with delayed reproduction at low food rations we frequently observed resorption of yolk from the ovaries. Yolk was accumulated during the first half of the intermoult period. Towards the end of the instar these substances disappeared from the ovary, to reappear in the next instar.

The proportion of total carbon investment allocated to the first brood was calculated for individual females (Table 2). It decreased significantly with food ration, but there was no difference between 50×10^5 and 30×10^5 cells/(ind×d), i.e. the intermediate food levels in the present studies. Allocation of carbon weight to body growth (G) and reproduction (R), as well as carbon loss to maintenance (M) during IM-2 were largest at the highest ration. No difference was found between the allocation patterns at the intermediate food levels. At 16×10^5 cells/(ind×d) but major differences were found between the early and the late group. Maintenance losses were enhanced in the late compared to the early group, owing to relatively large body size and a prolonged intermoult period in the former. This was counterbalanced by a decreased investment in growth. Hence, relative investment into reproduction during IM-2 was not affected by the time to maturation at this food ration.

Calculation of ingestion rates for animals in stage IM-2 indicated that diel fluctuations of food density owing to grazing were severe, except for ration 500×10^5 cells/(ind×d). After 24 h food concentrations were decreased by 76% to 99% at rations of 50×10^5 cells/(ind×d) and lower.

Table 2.

Food mediated carbon weight allocation to growth (G), reproduction (R) and maintenance (M) during IM-2. At the ration of $16 \cdot 10^5$ cells/(ind·d) 'early' and 'late' breeders are distinguished.

food ration (10^5 cells /(ind·d))		mean food conc. (mg C/l)	n	weight IM-2 (µg C)	weight IM-3 (µg C)	instar duration IM-2 (d)	G (µg C)	R (µg C)	M (µg C)	R $(G+R+M)^{-1}$
500		6.4	20	52.3	101.4	2.0	49.2^c	49.7^c	39.8^c	0.36^c
50		0.41	20	34.7	46.3	1.9	11.6^b	9.8^b	18.7^a	0.25^b
30		0.21	19	30.5	39.0	2.8	8.6^b	7.9^{ab}	18.4^a	0.23^b
16	early	0.09	11	29.6	38.9	3.9	9.3^b	4.1^a	20.2^a	0.12^a
	late	0.06	6†	46.5	51.8	4.2	5.3^a	6.0^{ab}	29.0^b	0.15^a
7		0.03	1	33.4	35.3	4.0	1.9	2.2*	17.8	0.10

Values indicated by the same letter do not differ significantly (Student-Newman-Keuls test, $P<0.01$).

†One length measurement has been lost.

*Neonate was born prematurely, hence weight (which is calculated from body length) is underestimated.

At the highest food level all females produced a second clutch in the instar succeeding the release of the first brood. However, at lower rations some animals had an empty brood sac during one or more instars. This increased with decreasing ration, i.e. 13%(50×10^5 cells/(ind×d)), 26% (30×10^5 cells/(ind×d)) and 100% (16×10^5 cells/(ind×d)).

Survival of progeny at starvation

Median survival time of newborn progeny during starvation was significantly affected by maternal food level. It increased with decreasing ration, yet no further increase was observed below 30×10^5 cells/(ind×d) (Table 1). Pooled individual survival times were positively correlated with body size ($r^2=0.16$, $P<0.01$, $n=71$). However, within treatment regression coefficients were variable, hindering further statistical analysis. Of the neonates from the highest maternal ration, 37% died during the first juvenile instar, and the remaining animals died in the second instar. In the other groups equal numbers of animals died during the second and the third instar. From the present data no relationship could be determined between neonate energy reserves and survival time. Most lipid index measurements were not carried out until some time after birth, yielding understimations of energy reserves obtained from the mother.

Combined effects of lead and food level on maturation

Daphnia exposed to 3.0 mg Pb/ l died within 70 h, irrespective of food level. At lower concentrations of lead, mortality did not rise above 10% during the experiment. The experiments were terminated after the release of the first brood, i.e. day 10 at the high food level, and between days 12 and 21 at the low ration.

Table 3.

Effects of lead exposure and food ration on maturation and production of the first clutch. Mean values for mothers and young are given.

			primiparae				progeny	
			IM-2		IM-3			
food ration (10^5 cells /(ind·d))	Pb conc. (mg/ l)	*n*	age (h)	body length (mm)	age (h)	body length (mm)	brood size	body length (mm)
160	control	8	107	2.78	155	3.23	10.5	0.92
	0.09	9	109	2.82	155	3.24	8.2	0.93
	0.27	10	107	2.74	155	3.18	9.5	0.92
	0.92	9	112	2.64*	160	3.06*	8.9	0.89*
	3.0	0	-	-	-	-	-	-
23	control	10	179	2.29	253	2.50	1.1	1.00
	0.08	8	194	2.28	265	2.47	1.0	1.00
	0.26	8	202	2.32	283	2.49	1.0	1.02
	0.90	8	289*	2.41	368*	2.55	0.6*	0.99
	3.0	0	-	-	-	-	-	-

*Lowest observed effect concentration (Dunnett's test, $P<0.05$).

The effects of lead on maturation and production of the first brood are shown in Table 3. Lowest observed effect concentrations (LOEC) were calculated for each food level separately. The LOEC was similar at both food levels; i.e. 0.92 mg Pb/ l. However, the sensitivity of maturation parameters to lead was different. Body length at IM-2 and IM-3 as well as size of young, were affected at the high ration. At the low ration the same concentration of lead caused a further delay of ovary provisioning and egg deposition, and a reduction in the brood size. The latter was owing to abortion of eggs and reduced viability of embryos. Egg deposition (IM-3) was shifted towards higher instar numbers at increasing concentrations of lead (Fig. 4). At high ration no such effect was found. Eggs appeared during the fifth instar, independently of toxicant concentration.

Interaction of food level and exposure to lead was explored with two-way analysis of variance. A significant interaction ($P<0.001$) was found with respect to size and age at egg deposition, but not for brood size and neonate length.

During IM-2 mass allocation of carbon to growth and reproduction was not affected by lead at the high food ration (Table 4). However, decreased investment into maintenance occurred at 0.92 mg Pb/ l, owing to smaller body size. At low rations investment in growth was significantly reduced at 0.92 mg Pb/ l. The amount of carbon spent on egg production was equal at all toxicant concentrations, but the number of eggs may be an unreliable indication of the number of viable young (above). The allocation to reproduction was not affected by lead at both rations. However, our calculations of maintenance might not be reliable in the presence of lead, as changes in costs owing to toxicant action were not taken into account.

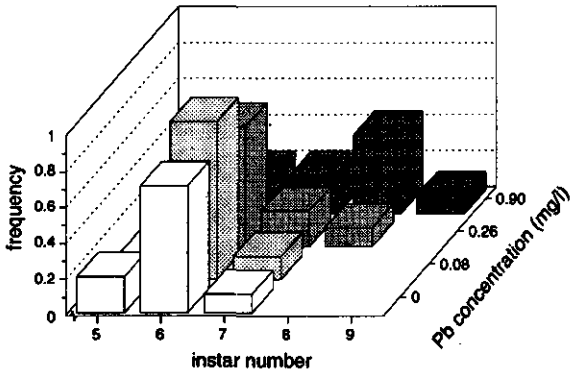

Fig. 4.
Effect of lead on egg deposition at low food. The instar number in which the first eggs occur (IM-3) is plotted on the X-axis.

From a comparison between the food experiment and the control groups in the experiment with lead, it was apparent that some between-test variation occurred. For instance, threshold sizes for maturation (*sensu* Ebert, 1992) were 1.60 mm and 1.84 mm respectively. Hence, it is not possible to extrapolate the results of one test to the other, even within a laboratory. Small environmental differences, prenatal as well as postnatal, modify the maturation process.

Table 4.
Effects of lead exposure and food ration on carbon weight allocation of primiparae into growth (*G*), reproduction (*R*) and maintenance (*M*) during

food ration (10^5 cells /(ind·d))	mean food conc. (mg C/l)	Pb conc. (mg/l)	*n*	weight IM-2 (µg C)	weight IM-3 (µg C)	instar duration IM-2 (d)	*G* (µg C)	*R* (µg C)	*M* (µg C)	$R(G+R+M)^{-1}$
160	1.7	control	8	59.5	86.0	2.0	26.5	43.2	37.1	0.40
		0.09	9	61.9	86.8	1.9	24.9	36.7	36.7	0.37
		0.27	10	57.8	83.2	2.0	25.4	37.7	35.9	0.37
		0.92	9	49.8	74.4	2.0	24.6	33.4	31.6*	0.37
		3.0	0	-	-	-	-	-	-	-
23	0.12	control	10	37.3	46.1	3.1	8.76	5.36	20.7	0.15
		0.08	8	36.9	44.7	3.0	7.75	4.87	19.3	0.15
		0.26	8	38.5	45.6	3.4	7.08	5.11	23.0	0.15
		0.90	8	42.4	48.5	3.3	6.11*	5.35	24.6	0.18
		3.0	0	-	-	-	-	-	-	-

*Lowest observed effect concentration (Dunnett's test, $P<0.05$)

Discussion

Effects of food level on maturation

D. magna displayed a wide variety of size and age at maturity in response to food availability. Moreover, our observations only represent a part of the full set of phenotypes that make up a norm of reaction for a given genotype. Other environmental factors, such as temperature (Perrin, 1988), food quality (Razouls *et al.*, 1991), maternal condition (Lynch & Ennis, 1983) and related factors, such as size at birth (Ebert, 1994), may cause additional variation. These have been controlled for in the present studies.

A large part of the observed variation in age and size at successive stages of the maturation process is closely bound up with growth rate. High growth rates, which occur at high food levels, speed up the developmental rate, but not beyond a maximum. This is probably owing to the functional response in *Daphnia*, which approaches a maximum value above the incipient limiting level (ILL) (McCauley *et al.*, 1990a). In our studies the ILL was between 50×10^5 and 500×10^5 cells/(ind×d). Food levels exceeding the latter did not increase the developmental rate further, as observed in a pilot experiment. A minimum age and a maximum size at IM-2 (ovary provisioning) and IM-3 (egg deposition) occurred at the highest experimental food level. As rations decreased to 30×10^5 cells/(ind×d) a gradual increase in age and a decrease in size at both IM-2 and IM-3 were observed; cf. Perrin (1989) and Lynch (1989), who found a critical, food independent size for IM-2 in *S. vetulus* and *D. pulex* respectively. Our results also conflict with a fixed size threshold at IM-3, which was assumed in the *Daphnia* model of Kooijman (1986). However, Ebert (1992) found an effect of food ration on age and length at IM-2 and IM-3 in *D. magna* similar to our studies. As the initiation of reproduction occurs during IM-1, i.e. oogenesis and the appearance of lipid droplets, Ebert argued that the decision to start reproduction was taken at the end of the preceeding instar (IM-0). This decision was marked by a threshold body length of 1.71 mm in Ebert's experiments. At low growth rates or small neonate lengths it takes more instars to reach the threshold, but the threshold length itself is independent of food level.

Eberts' model is only partly confirmed by the results of the present study. Indeed, a threshold size for the start of reproduction seems to occur at rations of 500×10^5, 50×10^5 and 30×10^5 cells/(ind×d). However, the occurrence of early and late reproduction at 16×10^5 cells/(ind×d) cannot be explained by a size threshold alone. The production of one egg during the sixth instar (early group) correctly follows the model, if a threshold length of 1.60 mm is assumed. The behaviour of the females that suspend egg production until the eighth instar, or even later (late group) is puzzling, because both groups grew at the same rate and passed the threshold at the same instar. Differences in feeding and production efficiency are therefore unlikely. Hence, variation in the distribution of energy between growth and reproduction seems to be responsible for the observed phenomenon. Apparently, 16×10^5 cells/(ind×d) represents a threshold food ration for reproduction under the present conditions. During the fifth instar both groups are able to ingest 95% of the daily ration, i.e. 10 μg C/(ind×d). The exact allocation of assimilated energy, which may be determined by hormonal balances, decides whether or not an egg is produced. Probably, females

in the early and late group matured in the same instar in a physiological sense, i.e. were capable of reproduction. Repeated allocation of yolk to and withdrawal from the ovaries, which was observed in the latter group, suggests that oocytes were already present several instars before eggs eventually developed and appeared in the brood chamber. Unfortunately, the presence and development of oocytes could not be perceived with the method of observation used in the present study. Histological examinations may provide additional information.

The observed pattern of yolk accumulation in the ovaries at the beginning of an instar and subsequent resorption as the female approaches moulting, suggests hormonal interference. Ecdysteroids, which seem to play a major role in the moult cycle and vitellogenesis (Bodar *et al.*, 1990), may regulate this phenomenon. Bradley *et al.* (1991) suggested that the production of a new carapace increases energy demands towards the end of an instar. From starvation experiments during IM-2 they concluded that provisioning of eggs takes place in the first half of the instar. If starvation was restricted to the second half of IM-2, clutch size was not affected; i.e. no material was drawn from the ovaries. Apparently, this result is not in line with our observations of yolk resorption. However, Bradley's animals were very well fed before entering starvation (clutch size was 55 eggs approximately). Hence, it can be assumed that female body reserves were sufficient to meet the energy demands at food scarcity, unlike the present studies. Our observations suggest that energy allocation to the ovaries is reversible. If maintenance needs, including the production of the new carapace, exceed the amount of energy stored elsewhere plus the energy intake from food, the deficiency is supplied from the reproductive organ.

As yet mathematical models of *Daphnia* do not allow redistribution of material allocated to reproduction (Hanstveit *et al.*, 1987; Gurney *et al.*, 1990).

Consequences of allocation strategies

From the present experiments it is concluded that investment into the first brood decreases with food availability. This is not in line with the assumption of Kooijman (1986): the fraction of assimilated energy allocated to reproduction is independent of food level, except for starvation conditions. However, the experiments of McCauley *et al.* (1990a) show a decrease in the proportion of energy allocated to reproduction with food level, in agreement with the present studies. The differences between high and low food rations became even more apparent at subsequent brood numbers. Incorporation of a food-dependent fraction to reproduction into their *Daphnia* model improved its predictive power (Gurney *et al.*, 1990).

Kooijman (1986) defined reproduction as eggs plus overhead, i.e. the amount of energy channelled to reproduction that is not incorporated into the eggs. However, this overhead is included in the maintenance term - through respiration - in the present study. Assuming a positive correlation between the mass of a brood and the amount of overhead energy, the difference between the proportion of energy allocated to reproduction at high and low food levels becomes even larger.

The calculation of carbon investment into growth, reproduction and maintenance revealed a modified allocation with delayed reproduction at 16×10^5 cells/(ind×d). Compared to early reproduction, carbon loss to maintenance was increased at the expense of growth, thereby saving the investment in reproduction.

In wild populations of *D. magna*, brood sizes may range from one to more than 60 (Green, 1956). Small clutches, caused by food limitation, may occur over long periods of the year (Lampert, 1978). The treatments in the present experiments, excluding the highest food ration, are representative of such conditions. In agreement with previous studies (e.g. Enserink *et al.*, 1990) both brood size and body length of progeny were affected by food level. Large broods consisting of small neonates were produced at high food levels and small broods with heavy, fat neonates occurred at low food. This strategy may improve survival of neonates at low food conditions, and increase population growth rate at abundant food. According to Ebert (1994) length at birth is a major factor determining age and length at maturity. With increasing food levels the effect on age at maturity decreases, whereas the effect on size at maturity increases. In addition, a positive correlation was found between length at maturity and the size of the first clutch. Hence, this reproductive strategy may also affect the process of maturation and, especially at high food levels, the number of progeny in the next generation.

With respect to the production of the first brood, within-environment trade-offs were only observed at 16×10^5 cells/(ind×d). At the lowest ration (7×10^5 cells/(ind×d)) almost no eggs were produced and at higher rations all females deposited their first clutch during the fifth instar. Within a given food level, the cost of delayed maturation should be compensated for by improvement of some other fitness trait, if this behaviour has any evolutionary significance. For instance, large primiparae might produce more progeny of a higher quality.

At 16×10^5 cells/(ind×d) most animals produced one egg in the first clutch, regardless of age and size at maturation. None of the 18 females produced a second clutch in the next instar. Whereas the number of progeny in 'early' and 'delayed' broods was equal, the body size of neonates in late broods was significantly larger than in early broods. In general, the ability to grow into the next instar under starvation conditions increases with neonate size (Enserink *et al.*, 1993). This was confirmed in the present study, but no difference was found between progeny of early and delayed broods produced at 16×10^5 cells/(ind×d). Hence, the possible advantage of producing a large neonate in a delayed brood was not confirmed in the present experiment. Improved fecundity at delayed maturation was observed by McCauley *et al.* (1990a) and Ebert (1991). In their studies within-environment trade-offs also occurred at higher food levels, unlike in our studies.

An attempt has been made to formulate a functional explanation for increase of neonate size with increased time to first clutch. For a tentative mechanistic explanation physiological restrictions are considered. According to Lei & Clifford (1974) larger, older females produce larger eggs which require a longer developmental time. Furthermore, instar duration generally increases during the five to six instars that follow the primiparous instar (Frey & Hann, 1985). Instar duration and egg size seem to be positively correlated. If only one egg is produced a larger intermoult period allows more time for egg provisioning, hence egg size increases (see also Ebert, 1993). Since delayed reproduction at 16×10^5 cells/(ind×d) coincided with increased instar duration, the above might be an explanation for larger egg sizes in this group.

Regarding the second clutch, a trade-off between growth and reproduction was observed at the intermediate rations, i.e. 50×10^5 and 30×10^5 cells/(ind×d). Some animals postponed the production of a second clutch. Apparently, these animals needed at least one instar to accumulate

new resources. Similar observations were made at the lower food levels. At 16×10^5 cells/(ind×d) even size increase was halted, following deposition of the first clutch. These phenomena support the conception of McCauley *et al.* (1990b), who proposed that individuals give priority to recovery of body mass over reproduction if their mass is less than a hypothetical mass-for-length, i.e. the body mass of a non-starved *Daphnia*. Individual variation with respect to food utilization may explain differences at a given food ration.

Effects of lead on the maturation process

The experiments with lead at two food levels showed an interaction between these factors, indicating that toxic effects on maturation are modified by food level. Some effects of lead toxicity were similar to increased food stress, suggesting increased energy demands for detoxification, tissue repair, etc.. At the high food level, which was representative of standardized toxicity tests, body growth was reduced by lead, but not to the extent that maturation was delayed. The size of primiparae decreased and, probably as a result, smaller neonates were produced. Exposure to lead also reduced body growth at low food. Under these circumstances, however, more instars to maturation were observed, which can be explained by the size threshold model of Ebert (1992). In addition, increased individual variation with respect to the number of 'pre-mature' instars occurred. This phenomenon resembles the results of the food experiment at the ration of 16×10^5 cells/(ind×d). Hence, the effect of lead on maturation is comparable to decreasing the amount of food. However, trade-offs in response to lead may be less efficient, as they are most probably not a result of evolution. Mortality of eggs and malformation of young owing to lead were observed at low food rations, indicating an effect on egg production or embryo development.

Similar interactions between effects of metals on maturation and the quality or quantity of food have been reported by some authors. In the experiments on *D. magna* by Winner *et al.* (1977) maturation was delayed in the presence of copper only with low-quality food. Chandini (1989) observed interactions between food level and cadmium toxicity to age and size at first reproduction in *D. carinata*. Toxic effects were more pronounced at low food levels.

On the basis of our experiments with individual *D. magna* some tentative conclusions can be drawn regarding effects of lead on the population level. The instantaneous rate of population growth (r_m) is sensitive to age-specific survival, time to first reproduction and clutch size. When exposed to 0.9 mg Pb/ l the latter parameters are adversely affected at the low food level, but not at high food.

Conclusions

We have found evidence for trade-offs between growth and reproduction, which affect the maturation process. Especially at low rations, or a combination of low food and toxic stress, such mechanisms cause great individual variation. Mathematical models predicting only one phenotype for a given set of environmental conditions seem to miss a characteristic feature of *Daphnia* life history.

Lead interacts with the subtle allocation patterns that determine the maturation process, especially at low rations. Such effects are not observed in standard toxicity tests, which are routinely carried out at abundant food. This hampers the extrapolation of test results to field conditions.

Acknowledgements

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chapter 6

Competition between cohorts of juvenile *Daphnia magna*: a new experimental model*

Abstract

Competition between two cohorts of juvenile *Daphnia magna* Straus was studied in a newly developed culture system, which allowed the cohorts to share resources while separated. The cohorts came from consecutive broods of a common mother cohort. Two different feeding regimes were applied: continuous feeding and daily pulses. Through body growth the cohorts created a situation of stable grazing pressure, where mortality was balanced by further growth. The larger animals appeared somewhat better competitors at constant low food levels, whereas smaller animals performed best at fluctuating food. On the basis of model simulations by Kooijman *et al.* (1989) body lengths were expected to converge, causing synchronisation of reproduction and thereby internally generated population oscillations. However, the experiments showed almost parallel growth curves, i.e. body sizes did not converge. Under the present conditions this pattern is best described by a linear growth model for juveniles. Reproduction was substantially delayed and no indication of synchronisation was found.

Introduction

The study of resource competition between herbivorous zooplankters has drawn increasing attention during the last decades. The majority of the studies concern interspecific competition (Brooks & Dodson, 1965; Hall *et al.*, 1976; Goulden *et al.*, 1982; Smith & Cooper, 1982; Tillmann & Lampert, 1984; Gliwicz, 1990). In many of the concepts that have been proposed to explain competitive dominance (for a comprehensive review see Rothhaupt, 1990) body size plays a prominent role. Factors that are considered decisive to the outcome of competition, and have a strong relation with body size, are resistance to low food, resource partitioning, energy allocation strategies and susceptibility to predation. All of these may also apply to intraspecific competition. However, competitive interactions *within* a species have been considerably less studied, although the importance of understanding both types of competition has been stressed by several authors (Frank, 1952; Neill, 1975; Hessen, 1990).

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Obstacles in experimental research

In species with isometric growth, such as *Daphnia magna*, experimental studies on intraspecific competition are seriously hampered by the problem of tracing an individual or a cohort in a population. Some authors (Pratt, 1943; Neill, 1975) have tried to follow individual *Daphnia* by staining them with neutral red. However, the staining procedure (restaining each two to three days during 12 to 24 hours) impedes practical application, not to mention undesirable side-effects. Hence, present knowledge on intraspecific competition is mainly derived from studies with cohorts and single species populations, where no special efforts have been made to track individuals or cohorts. The cohort studies consider only a special case of competition, i.e. between animals of the same age (e.g. Frank, 1952). In population studies competition between all size classes occurs, but only indirect evidence can be obtained regarding the life histories of the separate individuals and cohorts (e.g. Frank, 1952; Threlkeld, 1976; Lynch, 1978; McCauley & Murdoch, 1987). Development of an experimental set-up which enables the study of competition mechanisms may stimulate progress in this field.

Intraspecific competition and population dynamics

As a result of the interaction between *Daphnia* and its algal food supply, oscillations are common in both laboratory and field populations. According to McCauley & Murdoch (1987) such cycles can be internally generated by dominance and suppression of competing cohorts. In oscillating laboratory populations density peaks consist mainly of young, which are the offspring of adults born during the preceding peak. Hence, the cycle period corresponds with the generation time, which is quite long as a result of competition for food. For the same reason, reproduction is suppressed and it only recovers when the density of the cohort has declined owing to mortality.

Similar single-generation cycles are presented in Kooijman *et al.* (1989), which have been generated by a mathematical model based on the physiological properties of individual *Daphnia* (Kooijman, 1986). This approach enables the study of causal mechanisms on the level of the individual. According to Kooijman *et al.* (1989) synchronisation of life cycles is the main cause of internally driven oscillations. This prediction is based on a model assumption, i.e. ultimate size of an individual depends on food density, which in turn is depressed by population density. Individuals which are smaller than the ultimate size continue to grow, whereas body growth is suspended by those bigger than this size. This mechanism leads to convergence of body lengths. Reproduction is closely synchronised owing to a second model assumption, i.e. maturation occurs at a fixed body size. However, the modelled synchronisation is too severe, when compared to the behaviour of 'real' laboratory populations (Kooijman *et al.*, 1989; Van der Hoeven, 1989).

In order to understand this discrepancy, we have to examine the assumptions of Kooijman's model (1986) in more detail. Central to this issue is the relation between body size and assimilation or respiration. The model assumes that food intake is proportional to surface (~length^2) and maintenance costs are proportional to weight (~length^3). As a consequence, the rate of body growth declines as the animal approaches the ultimate size (cf.Von Bertalanffy, 1969, metabolic type I). This type of growth is often observed in growth experiments with individual *Daphnia* (Kooijman, 1986; Geller, 1987).

Daphnia growth models (e.g. Sinko & Streifer, 1969; Paloheimo *et al.*, 1982; Kooijman, 1986; Nisbet *et al.*, 1989; Hallam *et al.*, 1990) assume a certain relation between food level and the animal's growth rate. This relationship is based on theoretical considerations and experimental data. However, model assumptions derived from experimental results often do not take into full account the complexity of the environmental conditions. As a result serious extrapolation errors can occur when the simulated environment does not match the original. This problem may not in the least apply to the use of growth curves, as these are strongly affected by environmental conditions.

In the present study juvenile size convergence and synchronisation of first reproduction in *D. magna* populations are evaluated experimentally. The prediction of Kooijman *et al.* (1989) is adopted as a null hypothesis: body lengths of juveniles converge under conditions of intense competition for food. A culture system was developed to simulate food dynamics and competition during a density peak and succeeding decline phase of a laboratory population. This system allowed two cohorts with different mean body size to share the same food conditions, while separated. Two feeding regimes were applied, i.e. constant feeding and daily pulses, to examine the effect of resource variability. The results are discussed in comparison with alternative models.

Material and methods

Experimental conditions

The experiments were carried out with *D. magna* from our laboratory stock. The stock culture was held on Lake IJssel water, that was aerated and 0.45 μm filtered prior to use. In the experiments a 1:1 mixture of Dutch Standard Water (NPR 6503, 1980) and Lake IJssel water was used. Hardness of this medium was 217 mg/l (as $CaCO_3$), dissolved organic carbon (DOC) content was 2.8 mg C/l and pH was 7.8. A temperature of 20 ± 1 °C and a 13-h photoperiod were used. While the competition experiments were carried out, the temperature changed unintentionally (Table 1). *Daphnia* were fed green algae (*Chlorella pyrenoidosa* Chick), that were batch-cultured according to Dutch guidelines (NPR 6503, 1980). In order to obtain a stable food quality, the algae were harvested in the exponential growth phase, concentrated to approximately 10^{11} cells/l and stored in a dark room at 5 °C for one week at most. Rations were checked by means of a Coulter Counter. The maternal generation received 10^8 algal cells/(ind×d) approximately. The mean carbon content of a *C. pyrenoidosa* cell was 7×10^{-6} μg in our laboratory.

Competition experiments

Competition between two cohorts of *D. magna* was measured in a culture system (Fig. 1) in which the cohorts were separated, with the medium circulating between them. Fig. 2 gives a schematic representation of this system. It is composed of two interconnected glass vessels that contain 1.5 l medium each. A vessel consists of an inner and an outer tank, the latter being slightly wider. The cohorts are retained within the inner tanks by means of nylon gauze (mesh 300 μm) at the bottom.

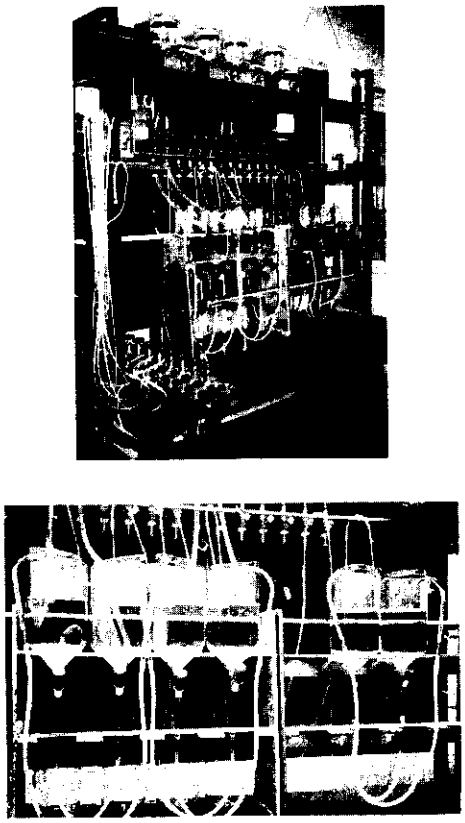


Fig. 1.
Photographs of experimental system with three pairs of culture vessels. From left to right are shown two experiments with continuous feeding and one experiment with pulsed feeding. Top picture: overview, with water column at the left side. Bottom picture: close up of culture vessels.

Through a silicon tube (8 mm ID), with a bent glass pipette at the end (3 mm smallest ID), medium plus algae is transported from the bottom outlet of the outer tank to the water surface of the paired vessel. The flow is driven by compressed air, that is 0.25 µm filtered and saturated with distilled water before it enters the water circulation. For pressure control, the air flow is bifurcated. One branch leads to the system via two capillary pipettes. The other branch ends up in a glass pipette which is suspended in a 1.5 m water column. By adjusting its position, the flow of medium in the system can be regulated quite precisely.

Food can be added in two different ways: daily pulses (D) or continuous feeding (C). In the first set-up 1×10^8 *Chlorella* cells are pipetted daily into each vessel. For the second feeding regime, a suspension of algae is dosed automatically by solenoids (Gemü, Ingelfingen-Criesbach, Germany) that open when energized. They have wetted PVC parts and soft valves (Viton) which ensure tight closure. Regulated by an adjustable automatic interval timer, the valves open for a few seconds with

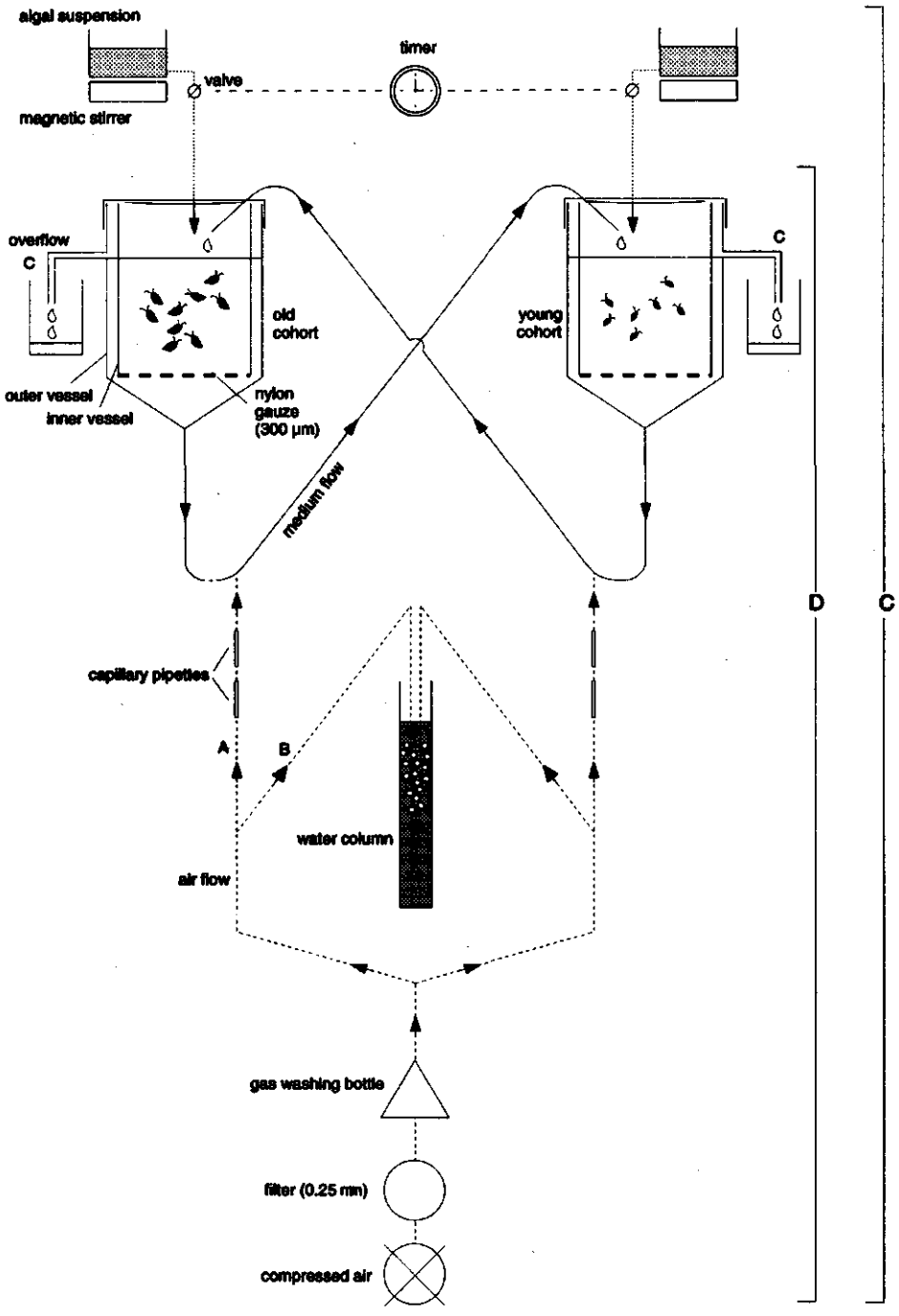


Fig. 2.
Schematic representation of culture system. D: experimental set-up for feeding regime with daily pulses, C: for continuous feeding. See text for further explanation.

a 30 minute interval. Via silicon tubing (10 mm ID) a total volume of *c.* 0.50 l algal suspension of known density is added daily. Surplus medium leaves the system via overflows situated in the cylinder wall of the outer vessels. The feeding suspension is freshly prepared each day. Sedimentation of algae in the reservoir is prevented by stirring slowly. The length of horizontal tubing is kept to a minimum for the same reason.

A very low food ration (initially *c.* 5×10^5 cells/(ind×d)) was used in order to create a situation of intense competition. Before test animals were introduced, the daily number of algae supplied to the system was checked in the experiments with continuous feeding. This ration is not only determined by density of feeding suspension, opening time of valves and number of pulses, but also by sedimentation of algae in tubes and valves. Regular cleaning measures improved the situation, yet we did not succeed to reproduce the daily food ration between experiments (Table 1). The medium was exchanged between the vessels with a rate of 3 to 10 l/h. For estimations of food level and mixing efficiency, 24 h recordings of algal density were carried out occasionally in the inner compartments of paired vessels during D and C experiments.

Table 1.
Test conditions of competition experiments with *D. magna*.

					start conditions		
feeding regime	code*	date	temp. (°C)	ration per cohort ($\cdot10^8$ cells/d)	diff. of age (d)	diff. of size (mm)	duration (d)
daily	D1	900802	20	1.0	3	0.30	145
	D2	910123	19	1.0	2	0.21	19
	D3	910222	23	1.0	3	0.39	41
continu.	C2	910123	19	0.60	2	0.20	19
	C3	910222	23	1.3	3	0.52	32
	C4	910226	23	0.80	3	0.40	37

*code: experiments indicated with the same code figure were run simultaneously, using the same neonate cohorts.

Two competing juvenile groups were used in each experiment, designated the 'old' and the 'young' cohort. They came from two consecutive broods (broodnumber >3) of a common mother cohort. At the start of the experiments they differed two to three days in age, and 0.20-0.52 mm in body length (Table 1). Each cohort initially consisted of 200 individuals. Both young and old cohorts were introduced to the test system as neonates (<24 h). During the first days of the experiments the old cohort competed with itself, i.e. a group of 200 individuals of the same age was introduced in each of the paired vessels. Soon after the birth of the young cohort, one of the old groups was replaced by 200 neonates. In experiment D1 however, a different procedure was followed. Prior to its introduction in the test system, the old cohort was held in a glass vessel and

given 10^8 cells/d. The water was changed daily. After the birth of the young cohort, both were held in the test system.

Surviving animals were counted and the inner vessels were rinsed two or three times a week. Dead animals and newborn young were removed. For the purpose of length measurement 20 individuals from each cohort were taken out haphazardly. Carapace lengths, measured from the top of the head to the base of the caudal spine, were determined with a dissecting microscope and a micrometer eyepiece. After measurement the animals were returned to the system. There was no indication of damage or mortality owing to handling. Carapace lengths of dead animals were also measured.

Calculation of size convergence

Size convergence of two competing cohorts was defined as a reduction in the difference between mean body lengths. Convergence or divergence was easily deduced from the observed growth curves. As a quantitative measure of convergence rate the half-time of the initial size difference ($T_{50,obs}$) was calculated from the length measurements. In addition, changes in size difference with time were determined with linear least-squares regression. Convergence is indicated by a negative regression coefficient.

A second approach was based on the Von Bertalanffy growth model (Von Bertalanffy, 1969, type I). Theoretically, individuals (or cohorts) following the same growth curve will approach the same ultimate length. The model for individual growth is given by:

$$L(t)=L_\infty-(L_\infty-L_b)e^{-\gamma(t-t_b)} \quad (1)$$

where: $L(t)$ = carapace length at time t (mm)
L_b = length at birth (mm)
L_∞ = asymptotic length (mm)
t_b = time at birth (d)
γ = rate constant of growth (d^{-1})

It was assumed that L_∞ and γ are constant as long as the mean individual food ration remains similar to the initial situation, i.e. if survival ≥75%. For each experiment these parameters were estimated for both cohorts simultaneously by non-linear least-squares regression. Since the parameters depend on food level, which is identical for competing cohorts, a common growth curve was calculated. L_b takes the value of the mean length at birth of both cohorts and is therefore treated as a constant in the calculations.

Following the same growth model, the length difference between cohorts, which differ with respect to age, at a given time t was calculated with:

$$L_{old}(t)-L_{young}(t)=(L_\infty-L_b)\times(e^{-\gamma(t-t_{b,young})}-e^{-\gamma(t-t_{b,old})}) \quad (2)$$

where: $L_{old}(t)$ = mean carapace length of old cohort at time t (mm)

$L_{young}(t)$ = mean carapace length of young cohort at time t (mm)

$t_{b,old}$ = time at birth of old cohort (d)

$t_{b,young}$ = time at birth of young cohort (d)

Using this equation the 'half-life' ($T_{50,model}$) of the initial size difference (at $t_{b,young}$) was calculated. When the size difference is reduced to 10% of its original value ($T_{10,model}$) the modelled size convergence is almost complete. Fig. 3 illustrates these calculations.

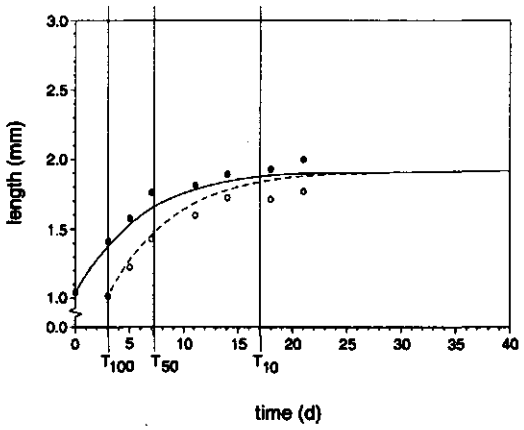


Fig. 3.
Calculation of size convergence rate based on Von Bertalanffy's type I growth curve, which serves as a null hypothesis in the present study. The markers represent mean cohort length as were measured in experiment D3 (closed symbols: old cohort, open symbols: young cohort). The curves are fitted according to equation (1). The decrease of the initial size difference (T_{100}) has been calculated with equation (2). See text for further explanation.

Grazing

The capacity of *Daphnia* to exploit its environment can be expressed by the filtration rate. At food concentrations below the incipient limiting level the filtration rate has a maximum value (Lampert, 1987). Retarded growth and reproduction in the present experiments indicate that this condition was met. Since a relationship between body size and filtration rate at the appropriate food conditions was not available, we used a relationship from Evers & Kooijman (1989) for *D. magna* at 20 °C and 5×10^8 *Scenedesmus subspicatus* cells/l:

$$F = 0.092L^2 \quad (3)$$

where: F = filtration rate (ml/h)

L = carapace length (mm)

This relationship was applied to calculate the filtration rate of a cohort, using the mean carapace length.

Results

Performance of the test system: a suitable design for competition experiments

The newly designed culture system proved to be a useful tool for competition studies. It enables detailed studies of the exploitation of a common food source by competitors that are similar in morphology, or should be kept apart for other reasons. Different, externally driven food dynamics can be simulated. With a few adaptations the system can easily be extended to hold three or more competing groups. The present design bears a slight resemblance to the flow-through system of Arndt *et al.* (1985), that was developed for the individual culture of copepods. They also used recirculating medium, that was drawn from the bottom of a common funnel and distributed between small culture vessels, which were suspended in the upper part of the funnel.

The reproducibility of test results was promising. It can be further improved by a better control of temperature and feeding, especially in the continuous feeding mode. Sedimentation of algae could be reasonably controlled, although not avoided. To some extent this hampered precise control of food dosage and it should be taken into consideration by the interpretation of experimental results. A further reduction of sedimentation areas, e.g. the corners of the container holding the food suspension, may increase accuracy.

Flow rates into paired vessels were not always exactly the same. Even small differences may occasionally result in unstable situations and overflow of one vessel at the worst. The flow of medium was very sensitive to the water levels in the column and in the gas washing bottle. Frequent checking and refilling was necessary, owing to water evaporation. For instance, a siphon connecting the outer compartments of paired vessels could correct for differences in flow rate.

A second reason to pay attention to the flow rate is that high rates may affect the condition of test animals. Adhesion of air bubbles to the carapace may increase locomotion costs, to compensate for increased buoyancy, or even cause mortality, which occurred in experiment C3. Thus, possible damage to test animals sets an upper limit to the flow rate.

Extensive exchange of medium and food between the vessels is of paramount importance when competitors are spatially separated. Fig. 4 illustrates the similarity of algal densities in paired vessels. These results are representative of all other 24 h recordings of algal density, which were carried out one to three times during each experiment (i.e. 14 recordings in total). On one occasion, half of the young cohort was removed from the system, to test whether or not equal densities of algae were the result of equal grazing rates of competing cohorts. Still, no difference was found between the vessels, indicating a satisfactory exchange of medium and food.

Daily addition of algae resulted in a peak density (*c.* 10^8 cells/l) which declined exponentially to a minimum of *c.* 5×10^6 cells/l (or 40 μg C/l) in 24 hours (Fig. 4). Continous addition resulted in a density of *c.* 10^7 cells/l (or 70 μg C/l) which was fairly constant, suggesting that grazing rate kept

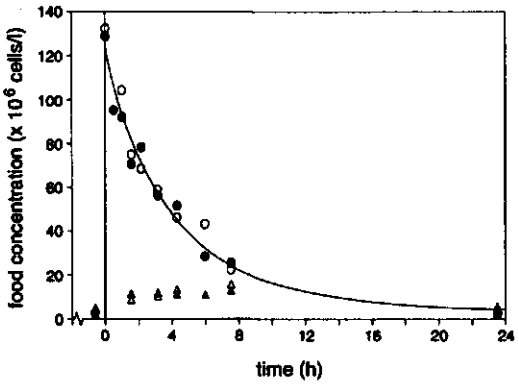


Fig. 4.
Daily fluctuations of food density in an experiment with daily feeding (D2, circles) and continuous feeding (C2, triangles). Measurements were done on the 8th day of the experiments. Vessel of old cohort, closed symbols; vessel of young cohort, open symbols. For experiment D2 analysis of covariance was carried out using ln-transformed food concentrations. Factor 'vessel' was non-significant. Pooled regression equation: $C(t) = 1166e^{0.248t} + 5.62$ ($r^2 = 0.96$).

pace with the supply of algae. The above carbon concentrations are in the same order of magnitude as the threshold food level, i.e. 60-80 μg C/l, as was determined by Kersting (1983) for adult *D. magna* (carapace length 2.5-3 mm).

In our experiments the medium remained surprisingly clear and no excessive growth of bacteria or algae was observed, even in the long-term experiment D1. This was probably owing to low food ration and removal of dead bodies.

Competition experiments

Throughout the experiments each cohort received a constant daily food ration. Undoubtedly, larger individuals took more of the common food supply than smaller *Daphnia*, owing to higher filtration rate. However, the results can be discussed in comparison with mean individual food ration.

As a consequence of thorough mixing of the medium, mortality in one cohort was beneficial to both competing cohorts. Mortality increased the amount of food available to each of the surviving *Daphnia*. This may stimulate body growth with a time lag of several days. According to Nisbet *et al.* (1989) increase in food level will be used for restoration of body mass if a *Daphnia* weighs less than the non-starvation weight-for-length. Hence, body growth also depends on the animal's feeding condition, which is a function of its life history.

From paired comparisons between the body length of a dead animal and the (interpolated) mean cohort length at the estimated time of death it was concluded that individuals smaller than the average length were more vulnerable to food shortage than bigger animals (t-test for paired comparisons, $n=326$, $P<0.001$).

For the interpretation of the experimental results, three important factors have to be considered, i.e. feeding mode, food ration (initial values as well as alterations during the experiment) and

temperature (cf. Table 1). In the following, the experiments are described in chronological order. In Fig. 5 an overview of the results is presented. Convergence rates and estimated Von Bertalanffy parameters are summarized in Table 2.

Table 2.
Growth curves and convergence rates according to Von Bertalanffy's type I model. For comparison the measured convergence rates are added.

exp. code	period (d)	regression coefficients n	l_0 (mm)	S.E.	L_∞ (mm)	S.E.	γ (d^{-1})	S.E.	r^2	convergence rates model T_{50} (d)	model T_{10} (d)	measured T_{50} (d)
D1	31	22	1.00	0	1.96	0.03	0.294	0.061	0.36	5	11	53
D2	19	15	0.98	0.02	1.93	0.09	0.163	0.042	0.89	6	16	>19
D3	21	15	1.03	0.02	1.92	0.06	0.164	0.029	0.94	7	17	25
C2	19	15	0.98	0.02	1.86	0.14	0.183	0.082	0.71	6	15	>19
C3	10	9	1.03	0.02	3.41	2.56	0.059	0.079	0.88	15	42	>32
C4	10	9	1.00	0.01	2.02	0.60	0.109	0.096	0.86	9	24	20

Experiment D1

In the first experiment growth of both cohorts was nearly linear. This corresponds to a gradual increase in mean individual food ration, which in turn is determined by mortality rate. Increased mortality, probably owing to senescense, occurred from day 110. One animal survived until day 148. Reproduction was substantially delayed. A few young were produced by the old cohort after day 79 (age 79 d) and by the young cohort after day 87 (age 84 d). The mean carapace length at maturation was 2.6 mm.

Mean body lengths of competing cohorts differed significantly up to day 79. The size difference decreased slowly, but significantly (r=-0.90, n=28, P<0.001). After 53 days the initial difference was reduced to 50% ($T_{50,obs.}$). Its half-life as predicted by the Von Bertalanffy model ($T_{50,model}$) was only five days.

Experiments D2 and C2

In these two experiments the feeding regimes were compared. In experiment D2, 35 *Daphnia* had disappeared on the fifth day. Since no carcasses were found within the vessels, it was regarded as an experimental accident, for instance overflow of medium. In C2 increased mortality in the young cohort occurred after 13 days, probably owing to starvation. Overall mortality in these experiments was relatively low, which is most probably related to the short duration. Hence, the mean individual food ration hardly changed.

The growth rate of the cohorts decreased after 10 days. In spite of the difference in daily food ration in these experiments, which was higher by a factor of 1.7 in D2, growth curves of the old cohorts were strikingly similar. The main difference between the two experiments occurred with

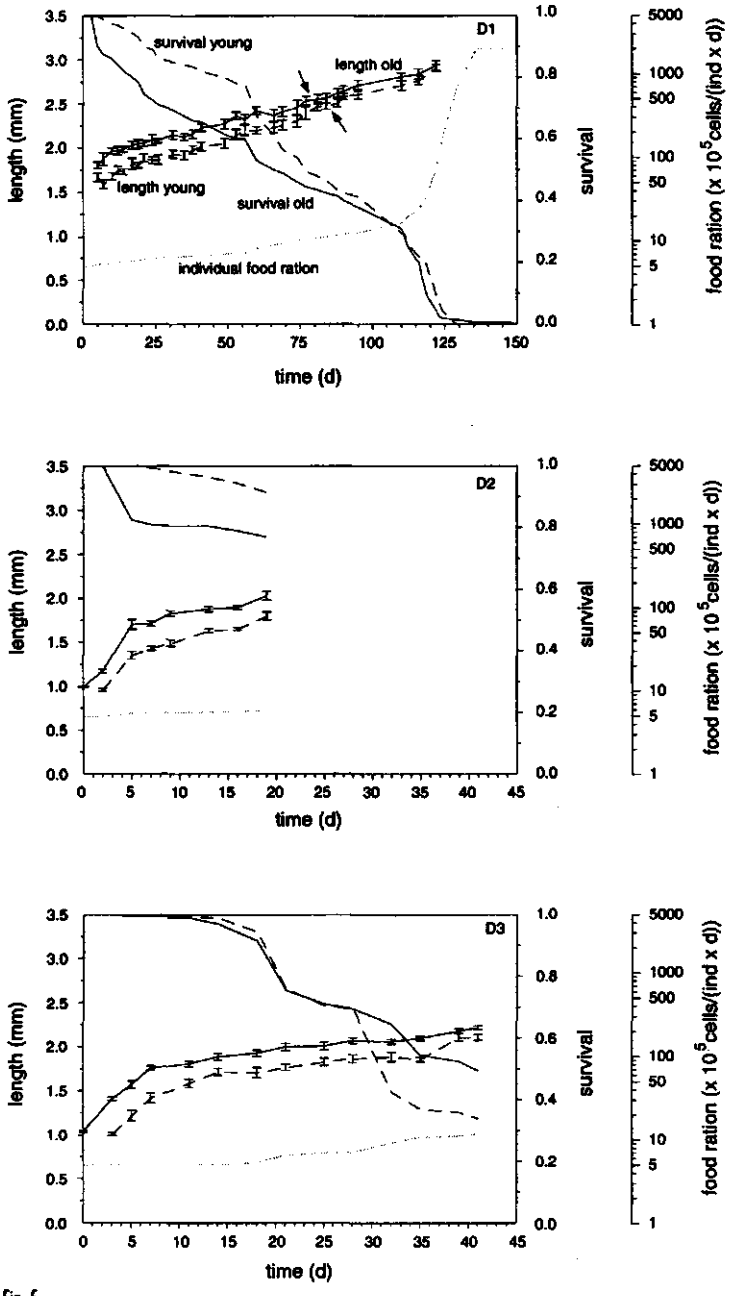
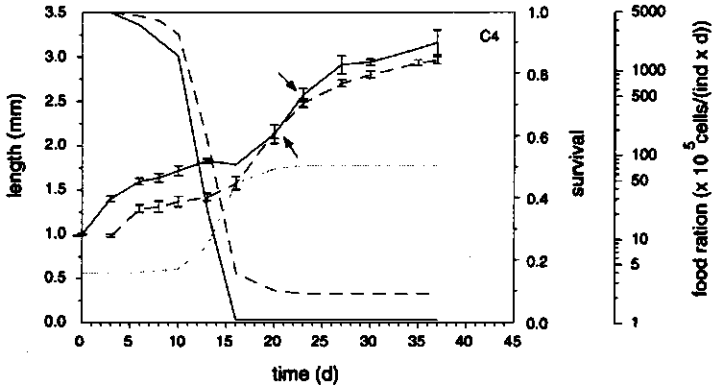
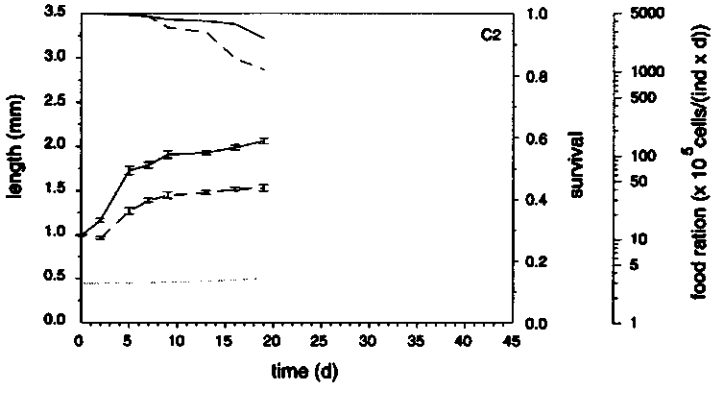
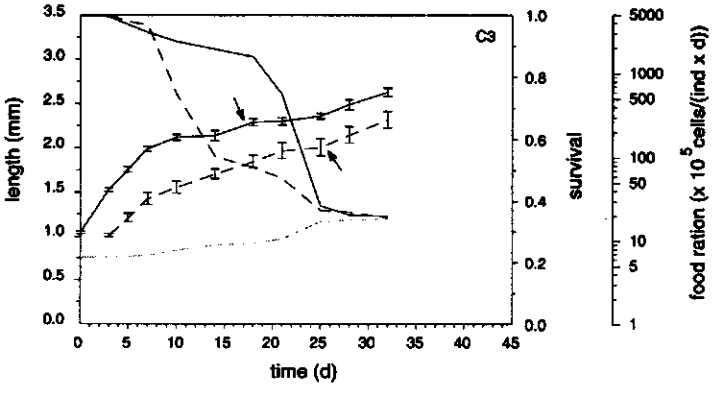


Fig. 5.
Growth, survival and individual food ration in all experiments. Error bars indicate 95% confidence limits for mean lengths. Old cohorts, drawn lines; young cohorts, dashed lines. Individual food ration (-●-) is defined as the total daily food ration given to both cohorts divided by the total number of survivors. Arrows indicate the birth of the first young. Note the different time scale of experiment D1.


C4
length (mm)
survival
food ration (x 10 5 cells/(ind x d))
time (d)


C2
length (mm)
survival
food ration (x 10 5 cells/(ind x d))
time (d)


C3
length (mm)
survival
food ration (x 10 5 cells/(ind x d))
time (d)

the performance of the young cohorts, where growth was significantly more depressed at the constant, low food levels of C2 than at the fluctuating levels of D2.

As a result, the size difference remained constant in C2 (r=0.73, n=6, n.s.), whereas a significant decrease (r=-0.85, n=6, P<0.05) was calculated for D2. However, it was not reduced beyond 80% of the initial size difference during the experiment (19 days). The modelled convergence rate did not agree with the observations, i.e. $T_{50,model}$ was six days in both experiments. No young were produced.

In spite of poor temperature control the results of experiment D2 were remarkably similar to the first 19 days of D1. This applies to growth and survival of both the old and the young cohort.

Experiments D3 and C3

Again, the experiment with daily feeding (D3) compares reasonably well to the previous studies (D1 and D2), except for a higher mortality rate during its second part. The resulting increase in mean individual food ration did not stimulate growth in this experiment compared to the growth curves in D1, apart from the last week (see below).

In the first two weeks size increments of the young cohort were consistently larger than those of the old cohort, causing some convergence of body lengths. Still, it took 25 days to halve the initial difference. In the last week the young cohort nearly caught up with the old cohort, probably as a response to a sudden increase in mean individual food ration some days earlier. A significant negative regression of size difference with time was found (r=-0.79, n=12, P<0.01). The modelled T_{50} was seven days. No young were born during this experiment.

The high initial food ration in experiment C3 profoundly stimulated body growth of the old cohort compared to all previous studies, whereas growth of the young cohort was similar to experiments C2 and D3. Mortality was high in C3, probably owing to the presence of air bubbles. The young cohort was substantially thinned out within two weeks, which was about 10 days earlier than in the old cohort. The pulses of mortality were followed by the growth curves, through an increase in mean individual food ration, with a time lag of *c.* three days.

Convergence was indicated by a significant negative regression (r=-0.94, n=9, P<0.001) of size difference. At the end of the experiment (day 32) a 40% reduction of initial size difference was found. The estimated $T_{50,model}$ again was too small, i.e. 15 days. The first young were born on day 17 in the old cohort and on day 25 in the young cohort. Mean body lengths were 2.3 and 2.0 mm respectively. From day 28 onwards reproduction was considerable and clutch sizes of one to eight eggs were recorded.

Experiment C4

The food ration used in this experiment was comparable to that of C2. However, the present experiment was carried out at a higher temperature. Growth curves of the young cohort were almost the same in both experiments, but in the old cohort growth was significantly depressed compared to C2. The majority of both cohorts died between day 10 and 16, probably owing to food shortage. A marked increase in mean individual food ration resulted, which was to the advantage of the few survivors.

The regression of size difference with time was non-significant (r=-0.55, n=11). During the first 10 days no size convergence occurred. From the remaining part of the growth curves no further conclusions can be drawn, because only a few animals were left. Again, the convergence rate was overestimated by the model ($T_{50,model}$=9 d). Reproduction started on day 20 in the young cohort and on day 23 in the old cohort. Mean body lengths were 2.2 and 2.6 mm respectively.

Grazing

Using equation (3) joint filtration rates of competing cohorts were calculated for each experiment. In Fig. 6 a general pattern can be recognized. Owing to body growth the filtration activity increased sharply during the first week. Thereafter, it levelled off at 100-120 ml/h, where body growth and mortality seems to be balanced, i.e. body growth is only possible if some animals die. Apparently, the cohorts existed near the carrying capacity. The pattern is disturbed when growth cannot compensate for serious mortality, as in experiments D3, C3 and C4.

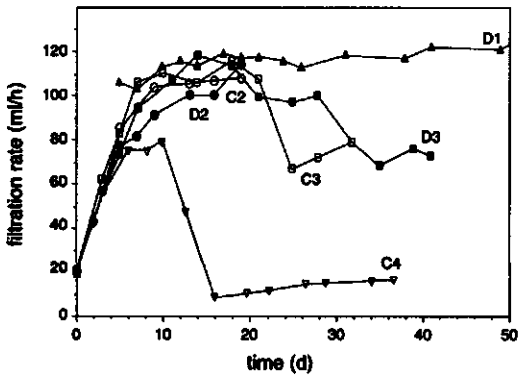


Fig. 6.
Development of grazing. Filtration rates of both cohorts have been summed for each experiment. They were calculated from mean body lengths and the number of surviving *Daphnia* (equation 3).

Discussion

Balance between juvenile growth and mortality under resource competition

The object of the present study was twofold. Firstly, development of a test system that enables the study of intraspecific competition. Secondly, experimental validation of body length convergence and synchronisation of reproduction in *D. magna*, which is regarded as an important cause of internally generated population oscillations (Kooijman *et al.*, 1989).

In our culture system conditions of intense competition for food could be created, which were comparable to conditions during a density peak and decline phase of a laboratory population with constant food supply. The paired juvenile cohorts that were kept in this system created a situation

of stable grazing pressure, which matched the constant daily food supply. The balance between body growth and mortality rate in these cohorts resembles the interdependence of birth and death rates in *Daphnia* populations which are in equilibrium with their food. In simple predator-prey models *Daphnia* density stabilizes at a level that can just remove algal productivity. If death rate increases, the algal density increases to yield a higher *Daphnia* birth rate, which compensates for the increased death rate and maintains the population at the original equilibrium density (McCauley *et al.*, 1988).

In the present studies two different feeding regimes were compared, i.e. daily pulses of food and continuous food addition. Davis & Alatalo (1992) also studied the effects of constant and intermittent food supply on growth and development of the copepod *Centropages typicus*. They found that daily fluctuations of food, up to 20-fold, could readily be integrated by this species. No differences were found in either growth or survival between pulsed and corresponding constant food treatments. In our studies however, slight differences between simultaneous experiments of both types were observed. In the second series, the total daily amount of food was somewhat lower in experiment C2 (constant food supply) than in D2 (daily pulses). Growth of the old cohorts was similar, but the young cohort grew faster at fluctuating food levels. In the third series, daily food ration was slightly higher in C3 than in D3. Initial growth of the old cohort was enhanced in the constant food treatment, whereas the young cohorts grew at the same rate. Hence, the competitive ability of the young cohort was better in the pulsed food treatment, whereas the old cohort dominated under constant food conditions. The latter observation agrees with Gliwicz's (1990) results: at constant food levels the threshold food concentration, at which assimilation equals respiration, was lower for large-bodied species than for small species within the family *Daphnidae*. We found this relationship also applicable to animals of the same species. Fluctuating food levels however, may favour other qualities, for instance the ability to withstand (short) periods of starvation.

In the experiments at 23 °C (D3, C3 and C4) mortality was higher than at 19 and 20 °C (experiments D1, D2 and C2). This effect was larger at constant than at fluctuating food levels. According to Neill (1981) temperature is correlated positively with mortality and negatively with growth at low food levels. He suggested that as temperature increases, metabolic needs for some nutrient materials increase faster than the ability of juvenile *Daphnia* to obtain them when food concentrations are low. Indeed, at higher temperatures growth rates were slightly lower than was expected on the basis of food level alone (experiments D3 and C4).

The reproducibility of the experiments with pulsed food supply was promising, whereas more variation occurred between experiments with continuous feeding. Here, we had some difficulty in establishing the purposed food ration, owing to sedimentation of algae in the tubes and valves of the dosing system. Such 'growing pains' can be overcome by more experimentation and refinement to the experimental set-up.

Convergence mechanisms

The outcome of competition within a population is determined by age- (or size-) specific attributes with regard to the collection of food and the allocation of energy to maintenance, growth and reproduction. The complexity of these interrelated processes is greatly reduced in the present studies, where competition is restricted to two non-reproducing cohorts. In this special case, low maintenance costs and efficient use of available energy for body growth are of vital importance. The result of these allocation strategies is reflected in the shape of the growth curve. From the results of the present experiments it is concluded that Von Bertalanffy's type I growth model (1969) considerably overestimates convergence rates of juvenile cohorts. Moreover, divergence of body lengths, which was also observed, conflicts with this model. Therefore, these results may have marked consequences for population simulations by *Daphnia* models that assume the Von Bertalanffy growth model.

The question arises why investigators have chosen this model to describe *Daphnia* growth from birth to senescence. Von Bertalanffy (1969) assumes that respiration rate is the limiting factor determining growth. He distinguishes three metabolic types, according to the relationship between body size and resting metabolism of animals. Respiration of type I is surface proportional. In type II it is weight proportional, and in type III it is intermediate between surface and weight proportionality. The exact value of the exponent in the allometric equation has been subject of numerous debates (Von Bertalanffy, 1969). According to Kooijman (1986) and McCauley *et al.* (1990) however, food intake limits growth rate. In food-limited environments, as in our studies, the latter assumption seems the most appropriate.

Although the limiting factor may change from respiration to ingestion, differentiation between metabolic types according to Von Bertalanffy remains meaningful. In the *Daphnia* models of Kooijman (1986) and McCauley *et al.* (1990) ingestion rate is proportional to body surface (type I). Measurements on *D. magna* by Evers & Kooijman (1989) confirmed this relationship. Yet Lampert (1987) preferred weight proportionality (type II). Nevertheless, he concluded that no general exponent of the length-ingestion rate relationship can be given, as it is affected by many environmental factors, among which are particle size and food concentration.

As published data are not conclusive, each of Von Bertalanffy's models may be applicable to *Daphnia*. Length increments may steadily decrease until a maximum size is attained (type I), or increase to form an exponential growth curve (type II). The growth curve of the intermediate metabolic type III, which is S-shaped, cannot be rejected either, since many authors scale ingestion rate of *Daphnia* between L^2 and L^3 (Lampert, 1987). Indeed, the type I model has been chosen by Taylor (1985; for adult growth only), Kooijman (1986) and Geller (1987), while Green (1956), Hallam *et al.* (1990) and McCauley *et al.* (1990) preferred the S-shaped curve, all on the basis of experimental data. Exponential growth for juvenile *D. magna* at different food levels was found by Tessier & Goulden (1987). Such variability may be caused by differences in environmental conditions and limiting factors. These might influence the relationship between body size and respiration or ingestion.

In addition to food intake the allocation of energy may also influence growth. Thus, juvenile and adult growth may have different characteristics. Many authors agree that the start of reproduction is reflected in the shape of the growth curve (e.g. Green, 1956; Lynch, 1980; Frey & Hann, 1985; McCauley *et al.*, 1990), possibly owing to changes in energy allocation patterns and/or energy uptake. According to Gurney *et al.* (1990) energy uptake in juveniles increases faster with size than maintenance costs, while in adults the reverse holds. This explains the preference of these authors for the S-shaped growth curve (cf. McCauley *et al.*, 1990).

In the first juvenile instars prenatal conditions further complicate the growth process, because maternally provided energy reserves add up to juvenile assimilate (McCauley *et al.*, 1990; Enserink *et al.*, 1993). The amount of maternal reserves in *D. magna* is highest in low food environments (Enserink *et al.*, 1990), which may cause a relatively large size increment in the first instar(s). At high food, maternal energy contributes only a little to juvenile growth rate. If food levels change between generations, which is often the case in laboratory experiments, the observed growth curves may be affected.

Results of growth experiments are, to a very large extent, modified by environmental conditions, especially food dynamics. If *Daphnia* are kept in ample volumes of water and are fed continuously (i.e. constant food level) juvenile body growth appears linear (Taylor, 1985) to exponential (Lampert, 1977) and the start of reproduction is the main factor which decreases growth rate. More often transfer cultures with constant food input are used. Here, growth rate is decreased by increasing food limitation and periodic starvation, i.e. by container size and feeding regime (e.g. Tillmann & Lampert, 1984; Gurney *et al.*, 1990).

Whereas growth models describe individual growth as a function of environmental conditions and the animal's life history, it should be noted that in the experimental search for 'correct' growth curves often little attention is given to the particular conditions of the experiments.

Food dynamics in our experiments resulted from constant food input and grazing pressure, which increased until cumulative ingestion rate approached food supply. Substantial mortality could improve food conditions. Reproduction was initiated when two conditions were met: individuals had passed a threshold size for maturation *and* food uptake was sufficient to produce at least one egg (cf. Enserink *et al.*, 1995). We can assume that larger *Daphnia* ate more food than their smaller sisters, owing to higher filtration rates. The growth curves of the old and young cohorts progressed almost parallel, in response to food conditions. Neither pronounced convergence, which indicates type I growth, nor strong divergence owing to exponential growth were found. These results suggest that a linear growth model can be used as a simple and appropriate description under the present conditions. In this model, small and large individuals grow at the same rate, i.e. dL/dt is constant, at a given food concentration. Changing food availability result in complex growth curves, as in the present experiments.

Consequences for population dynamics

In the present experiments convergence rates of juvenile cohorts were very low to nil under conditions of strong competition, which are comparable to the situation during a population peak

and succeeding decline phase. Besides, no indication was found for synchronisation of first reproduction. Therefore, synchronisation of life cycles within a population seems an unlikely cause of internally generated density oscillations.

Our results do not agree with the predictions of Kooijman *et al.* (1989), e.g. strong and persistent population oscillations owing to synchronisation. These authors aimed to improve the simulated population dynamics through the introduction of individual variability in ingestion rate. Increasing variation decreased both regularity and amplitude of the oscillations. However, sufficient reduction was only obtained if the coefficient of variation (CV) exceeded 20%. Indeed, variation can be considerable, especially at low food levels. For instance, the CV of age at maturation was 47% in a previous study (Enserink *et al.*, 1995). However, size at maturation was less variable, i.e. 7%. From this study it was concluded that the observed variation was caused by trade-offs between growth and reproduction and not by differences in ingestion rate. Van der Hoeven (1991) argued that a value of 20% is markedly greater than individual variation observed in experiments. Hence, variation in ingestion rate alone cannot explain the difference between modelled and experimental population dynamics.

On the basis of the present experiments, we propose reconsideration of a basic assumption of Kooijman's *Daphnia* model (1986), i.e. the Von Bertalanffy type I growth model (1969), in case individual growth is used as a basis for simulation of population dynamics. It should be noted that some adjustments in the model are trivial to the simulation of individual growth, while they may lead to radical changes in population behaviour. Parallel growth of competing individuals may contribute to the development of more realistic simulations.

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chapter 7

Summary and concluding remarks

The waterflea *Daphnia magna* is a widely used test organism in ecotoxicological studies. Acute and chronic laboratory tests yield basic information for the development of water quality standards, assessment of potential hazards of (new) chemicals, waste water licences and sanitation measures for contaminated sediments. Environmental risk assessment also includes extrapolation from laboratory to field, for which theoretical models are applied. Reliable results can only be obtained if:

- toxicity test results are both accurate and reproducible, and
- extrapolation models take account of major ecological processes.

It has been recognized that under current international guidelines for *Daphnia* toxicity tests interlaboratory variation of test results is disappointingly large (Cowgill, 1987; Baird *et al.*, 1989a). Differences in test conditions and culture techniques are regarded as a main source of variation, but their relative contributions are largely unknown.

The distance between a single-species test under laboratory conditions and the response of an aquatic ecosystem is enormous, in terms of complexity. A first step has been made by Kooijman, who developed a model to predict effects of toxicants on *Daphnia* populations from effects on individuals (Kooijman, 1986; Kooijman *et al.*, 1989). From comparisons between experimental and simulated population dynamics research questions regarding critical model assumptions were derived.

The present study focuses on food availability, which plays a central role in the life history of *D. magna*. Its development and reproduction are dependent on food level, as well as its sensitivity to toxic stress. The importance of food for the culture of test animals was investigated, as a contribution to standardisation of toxicity tests. Besides, assumed relationships between food level and *Daphnia* growth and reproduction are validated, in order to support development of extrapolation models. The experimental approach has been chosen to address the research questions related to both toxicity testing and extrapolation models. New techniques were developed for that purpose. Cadmium, chromium and lead, which are designated as priority pollutants by the International Rhine Committee (1987), have been applied as model substances. The investigations have been carried out at the Instute for Inland Water Management an Waste Water Treatment in Lelystad.

New experimental methods

Length measurements are basic to life history research. Therefore, electronic sizing of living *D. magna* was pursued by development of a computer program, which was based on an existing image

processing system. This method, which is described in *chapter 2*, was much more rapid than manual measurements with a microscope, although it was less precise. Image analysis is recommended for determination of large samples, e.g. length frequency distributions of populations. Debris in the size range of the objects has to be removed. When it comes to precision, for instance determination of growth curves, the manual method should be used.

Competition mechanisms within a *Daphnia* population were investigated with a newly developed culture system (*chapter 6*). It consisted of two interconnected culture vessels, each holding a cohort. An air-driven flow of medium plus algae circulated between these vessels, hence allowing the cohorts to share the same food conditions. The system proved to be a useful tool for competition studies. It enables detailed studies of the exploitation of a common food source by competitors that are similar in morphology, or should be kept apart for other reasons. Different, externally driven food dynamics can be simulated. With a few adaptations the system can easily be extended to hold three or more competing groups.

Standardization

Current international guidelines predominantly aim at standardisation of conditions during toxicity tests. Therefore, culture methods, i.e. pre-test conditions, differ among research laboratories. As an example, methods practised at the Institute for Inland Water Management and Waste Water Treatment (RIZA) are described in *chapter 2*. The experiments in the present study were conducted with *D. magna* cultered in natural water from Lake IJssel. The green alga *Chlorella pyrenoidosa* was used to feed the *Daphnia*. Transfer cultures were supplied with constant daily food rations, and young were removed three times a week. Hence, the density of algae varies with cumulative filtration rates of the mother cohort and the progeny which has not yet been removed. This may carry over into the results of ecotoxicological studies, as maternal feeding conditions can affect the quality of the young. Cowgill *et al.* (1985) showed an inverse relationship between brood size and weight of newborn *D. magna* in a laboratory population. They suggested the existence of a reproductive strategy, which earlier had been proposed by Hutchinson (1951) on the basis of field observations. According to this strategy females spread their genes around under favourable conditions by producing many small, 'cheap' neonates, while heavy, stress-resistent young are born when food is sparse. An important objective of the present thesis was to test the validity of this strategy and to explore ecotoxicological and ecological consequences by experimentation (*chapters 3 and 4*).

It could be demonstrated in our studies that reproduction of females, which were exposed to an abrupt increase or decrease of food ration between the third and the fourth brood, was fully adapted to the new situation in three instars, or *c.* eight days at 20°C (standard laboratory temperature). Brood sizes ranged from 7 to 76 and neonate carapace lengths from 1.1 to 0.92 mm. The smallest young (0.80 mm) were observed in the first brood. The latter is a well-known phenomenon that is accounted for in toxicity test guidelines (e.g. OECD, 1984) by discouraging the use of the first brood. The main reason for this advice is reduced survival in controls, which may invalidate the test (e.g. Cowgill *et al.*, 1986). The present study shows that large neonates contained more lipid

reserves than small ones. Therefore we may expect a positive correlation between survival at starvation and body size. This was confirmed in the starvation experiments described in *chapter 5* and Enserink (1989), but not in similar experiments in *chapter 4*. In connection with unfed 48 h toxicity tests it is worth noting that median survival times never fell below 4 d, even for the smallest (0.77 mm) neonates.

An even more important reason to pay attention to neonate size is that small neonates can be more sensitive to toxicants than large young in acute (48 h) tests (*chapter 3*). When exposed to cadmium there was a threefold difference between the LC_{50}'s for small and large animals. However, no such effect was found in toxicity tests with chromium(VI), i.e. LC_{50}'s were similar in all tests, which probably relates to different modes of action of these two metals.

In chronic (21 d) toxicity tests the initial size of test animals appeared to be less important (*chapter 4*). Whereas the trends observed in acute tests were consistent in several trials (e.g. Enserink, 1989), this was not the case in chronic tests. The results suggest that environmental conditions during the test mask subtle differences in neonate size and lipid reserves. The variation in effect concentrations caused by non-simultaneous replication was in the same order of magnitude, i.e. a factor of 2, as the effects of initial body size. Probably, food supply during these tests was inadequately controlled.

The most conspicuous conclusion of the present studies is that maternal food ration is of paramount importance to neonate fitness and hence to the results of acute toxicity tests, at least for some chemicals (Enserink, 1989; Enserink *et al.*, 1990). Similar results were obtained independently by Baird *et al.* (1989b). More recently, a number of other investigators have confirmed these findings.

The reproductive strategy in *D. magna* was also observed by Cox *et al.* (1992), Naylor *et al.* (1992) and Viganò (1993). According to Naylor *et al.* (1992) the inverse relationship between maternal food level and neonate size also holds for dry weight, although this parameter appeared to be more variable than body length. Even under normal culture conditions, when food level is not purposely varied, brood to brood oscillations of neonate weight and length were inversely related to oscillations of mean clutch size (Viganò, 1993; Lazorchak & Waller, 1993). Such variation can be caused by unstable quality or quantity of food supply, increased grazing capacity owing to body growth and periodic presence of neonates.

The results of the current studies suggest that the influence of maternal food conditions on the results of acute (48 h) toxicity tests depends on test substance. Cadmium toxicity was modified by neonate size, but the toxicity of chromium remained unaffected. The largest influence was reported for 3,4-dichloroaniline, i.e. a factor of 6 (Baird *et al.*, 1991). A significant positive correlation between neonate size and LC_{50} was found for sodium bromide and 3,4-dichloroaniline (Naylor *et al.*, 1992), and copper (Lazorchak & Waller, 1993). Viganò (1993) found no effect of neonate size on LC_{50}'s for ethylbenzene and *n*-butylbenzene, but the variation of test results was very limited, as was the size range of the test animals. For cadmium however, the effect of maternal ration was confirmed (Baird *et al.*, 1991), but neonate length showed no correlation with LC_{50} in the experiments of Naylor *et al.* (1992), which is inconsistent with the results of the present studies.

Several differences between our study and that of Naylor *et al.* (1992) might account for these results, e.g. the presence of food during the test, the use of another clone and a different test medium. It is of interest to note that both studies were carried out according to standard, but different, test protocols. Whereas maternal food ration, which was purposely varied, accounted for a within-laboratory variation of a factor 3 in the present study and the experiments of Baird *et al.* (1991), who worked at the same laboratory as Naylor *et al.* (1993), a 20-fold difference occurred between the laboratories. From the viewpoint of standardization, sources of variation within and between laboratories are equally important.

The significance of maternal food conditions for toxicity tests has been clearly demonstrated above. It is therefore recommended to include pre-test conditions in standardization programmes and test guidelines. Several other factors are known to be relevant, for instance feeding conditions during the test (Winner *et al.*, 1977; Chandini, 1988a, b; Soares, 1989; Lazorchak & Waller, 1993; Sims *et al.*, 1993; Klüttgen & Ratte, 1994), genotype (Soares, 1989; Baird *et al.*, 1991), medium (Winner, 1985) and even statistical evaluation of test results (Hoekstra, 1993). However, very little is known about their relative contributions and interactions. Ring-tests are excellent instruments to investigate such combined effects. At present, an international ring-test is conducted to improve the OECD guidelines (1984) for chronic toxicity tests, with respect to genotype, medium and feeding during the test. This investigation is coordinated by the University of Sheffield (UK), Department of Animal & Plant Sciences. In order to obtain a sufficient overview of sources of variation within and between laboratories and to identify the most important factors, further investigation is needed. Current guidelines do not garantee standardized test results in the strict sense of the word, which decreases the reliability of safe levels for water management and of bioassays conducted for regulatory purposes. Therefore, a collective decision should be made on:

- the desired quality of toxicity test results for water management and
- the amount of detail in culture and test protocols that is required to meet this quality.

Extrapolation

The phenotypic plasticity of *D. magna* in response to its food source is impressive. All major life history traits can adapt rapidly to food availability, which undoubtly has evolutionary significance as *Daphnia* experiences a nutritionally variable environment during its lifespan. The challenge to designers of simulation models is to describe and incorporate those relationships which are indispensable for attaining the goals of the model.

In the model of Kooijman (1986) for growth and development of individual *Daphnia*, a balance between mathematical simplicity and biological realism was pursued. Modelled individuals were aggregated into simulated single-species populations (Kooijman *et al.*, 1989; Van der Hoeven, 1991). In this way, physiological effects of toxic chemicals can be translated into population dynamics, which was regarded as a step towards modelling the response of ecosystems to toxic stress. Ecosystems contain many interacting populations. In order to avoid a complex tangle of detailed sub-models a collection of assumptions was produced, partly based on conceptions of physical mechanisms, partly on the premise that everything is extremely simple unless it proves to

be more complex and partly on empirical data. The idea was to strip details from the sub-models in the process towards the ecosystem model. The *Daphnia* model was regarded as a test case for a more general model, which should be applicable to many species (Kooijman *et al.*, 1987). Nevertheless, a certain amount of detail is nescessary for meaningful extrapolations to higher levels of organisation.

A comprehensive list of Kooijman's (1986) model assumptions at the level of the individual is given by Van der Hoeven (1991). In the present thesis a number of these assumptions was evaluated experimentally:

- neonate size is fixed;
- size-specific storage of a neonate is identical to that of its mother at the moment of egg formation;
- a minimum size is required for reproduction;
- a fixed portion of utilized energy is spent on reproduction and the remainder on growth and maintenance;
- ingestion rate is proportional to body surface;
- energy costs for maintenance are proportional to body weight.

An attempt was made to assess their relevance for extrapolation.

In *chapters 3 and 4* a reproductive strategy for *D. magna* in reponse to food availability is described. Body size and lipid content of neonates appeared to be inversely related to maternal food ration. Cox *et al.* (1992) observed no further decrease of neonate size when food was no longer restrictive, i.e. above the incipient limiting level. These findings do not agree with two model assumptions: size at birth is independent of food level and size-specific energy storage of a neonate is identical to the size-specific storage of its mother. The reproductive strategy mentioned above has important consequences for survival and development of neonates under low food conditions. Survival time at starvation can increase from 4 to 9 days with increasing neonate size (*chapter 5*). In addition, large and fat young can develop into their third instar without food (*chapter 4*). This may decrease time to first reproduction, as will be shown below.

In the model, deposition of the first clutch, i.e. the primiparous instar, is initiated as the female attains a certain body size, which is independent of food level. In *chapter 5* however, body sizes of primiparae differed significantly among food rations, with minimum sizes at intermediate rations. A threshold body length for maturation was found two instars earlier, i.e. just before the preadolescent instar, in concordance with Ebert (1992). However, this threshold was not completely independent of food. At low food rations trade-offs occurred between growth and reproduction. One group of animals delayed their first brood in favour of body growth. The young of these delayed broods were significantly larger than young that were born earlier at the same maternal food ration, probably owing to increased instar duration. It was concluded that a minimum energy requirement for reproduction was met at this food ration.

A remarkable phenomenon was observed at low food rations. Allocation of energy reserves, i.e. yolk, to the ovaries appeared reversible. Accumulation during the first half of an instar and redistribution towards the end of the instar suggested that energy needs for the production of a new carapace have priority over egg production. As yet mathematical models of *Daphnia* do not

allow redistribution of material allocated to reproduction (Hanstveit *et al.*, 1987; Gurney *et al.*, 1990).

From the distribution of biomass during the production of the first brood it was concluded that the proportion of energy allocated to reproduction increases with food availability. Similar results were obtained by McCauley *et al.* (1990), who improved the simulations of their *Daphnia* model by incorporating a food-dependent fraction to reproduction (Gurney *et al.*, 1990). In the model of Kooijman however, the fraction of assimilated energy allocated to reproduction is assumed constant, except for starvation conditions.

Combined effects of food ration and exposure to lead on maturation were also investigated in *chapter 5*. A significant interaction between food and lead concentration was observed, which means that toxic effects were dependent on food level. Body growth was reduced at both food levels, leading to smaller primiparae with smaller progeny at abundant food, whereas delayed maturation and egg mortality were observed at the low food ration. Except for egg mortality, the effects of lead on maturation resemble increased food stress. Similar observations have been made in experiments with copper (Winner *et al.*, 1977) and cadmium (Chandini, 1989; Klüttgen & Ratte, 1994). Extrapolation from chronic toxicity tests, which are normally carried out with large food supply, to low food environments can therefore generate wrong conclusions.

Density oscillations in laboratory populations with constant food supply are a common phenomenon. However, it is not clear whether such behaviour is mainly caused by intrinsic properties of the populations themselves or by experimental irregularities. On the basis of an analysis of published population behaviour, Van der Hoeven (1989) suggested that the latter option is very probable. Therefore, the simulations of the *Daphnia* model, which show regular, persistent oscillations, have been questioned (Kooijman *et al.*, 1989). The major driving force of these oscillations is synchronisation of life cycles during the decline phase of a population. This prediction mainly follows from the assumption that food intake is proportional to surface ($\sim \text{length}^2$) and maintenance costs are proportional to weight ($\sim \text{length}^3$). As a consequence, the rate of body growth declines as the animal approaches an ultimate size, which in turn is depressed at low food levels. Individuals which are smaller than the ultimate size continue to grow, whereas body growth is suspended by those which are larger than this size. This mechanism leads to convergence of body lengths. The assumed growth model is referred to as Von Bertalanffy's type I model (1969). Reproduction is closely synchronised owing to another assumption, i.e. first reproduction occurs at a fixed body size (cf. *chapter 5*).

Convergence and synchronisation among two juvenile cohorts was evaluated in an experimental set-up that simulated food dynamics and competition during a density peak and succeeding decline phase of a laboratory population (*chapter 6*). The cohorts, which came from consecutive broods of a common mother cohort, were separated while they shared the same food source. Two feeding regimes were used, i.e. constant food input and daily pulses, to examine effects of resource variability. Under the experimental conditions convergence was very slow or absent and no synchronisation of reproduction occurred. The larger animals performed best at constant low food levels, whereas the smaller cohort was a better competitor at fluctuating food. Modelled convergence rates, using the Von Bertalanffy model, were unrealistically high. Therefore, the

suitability of this model under the present conditions was discussed in comparison with other growth models. Parallel growth curves suggested a linear growth model for juvenile *D. magna*, although it was recognized that the shape of the growth curve is extremely dependent on the specific conditions of the study.

From the mechanisms described above it is concluded that close synchronisation of life cycles in laboratory populations and hence severe oscillations are not expected. Parallel growth, increased individual variation at low food level (cf. Cox *et al.*, 1992) and trade-offs with respect to first reproduction have a stabilizing effect. It has been shown that lipid reserves of mother and progeny are inversely related, which decreases the population growth rate and enhances the survival probability of neonates under resource limitation. In general, phenotypic plasticity, especially adaptation to low food, is underestimated in the present model assumptions. Simple models may perform satisfactory at the level of the individual, but they can lead to erroneous population dynamics. Incorporation of some more biology will undoubtly improve the model simulations and thereby the extrapolation of toxic effects from the individual to the population level. However, the balance between mathematical elegance and the full complexity of *Daphnia* is precarious. A stepwise incorporation of the most relevant life history strategies in combination with sensitivity analysis of the model could improve our understanding of population dynamics.

The results of *chapter 5* and adverse effects on growth and reproduction, which are observed for many toxicants (cf. *chapter 4* and Enserink *et al.*, 1991), suggest that toxic substances impair *Daphnia*'s capacity to efficiently exploit its food source. This could lead to increased densities of (edible) algae. However, effects on ecosystem level depend on many factors, e.g. relative susceptibility of competing zooplankton, predators and food organisms. For instance, Marshall & Mellinger (1980) observed both decreased and increased phytoplankton production in cadmium spiked enclosures of plankton communities. In the first case primary production was directly affected by the test substance and in the second case reduced zooplankton abundance compensated for toxic effects on the algae. In a review on pesticide stress in freshwater ecosystems Brock & Budde (1994) concluded that primary effects can be predicted from laboratory tests, if exposure of field populations can be estimated. However, secundary effects are unpredictable in most cases. Recently, Scholten *et al.* (1994) stated that eutrophication problems are enhanced by impaired zooplankton grazing, owing to the presence of toxic substances in surface water. Although their conclusions raised great controversy in The Netherlands, the above statement could serve as a valuable hypothesis in future investigations. At present, the influence of toxicants on aquatic ecosystems is largely unknown. Research on the combined effects of nutrients and micropollutants is certainly worth pursuing. Besides, it could stimulate further cooperation between ecologists and ecotoxicologists.

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Samenvatting en conclusies

De watervlo *Daphnia magna* is een bekend proefdier in de ecotoxicologie. De resultaten van acute (kortdurende) en chronische (langdurende) laboratoriumtesten met watervlooien worden toegepast in normstelling, toelatingsprocedures voor nieuwe chemicaliën, vergunningverlening voor afvalwater en sanering van verontreinigde waterbodems. Voor de vertaling van toxische effecten in het laboratorium naar risico's voor watersystemen zijn theoretische extrapolatiemodellen beschikbaar. Betrouwbare schattingen kunnen alleen worden verkregen als:

- de resultaten van toxiciteitstesten zowel nauwkeurig als reproduceerbaar zijn en
- extrapolatiemodellen rekening houden met de belangrijkste ecologische mechanismen.

Helaas garanderen de huidige internationale richtlijnen voor toxiciteitstesten met *Daphnia* niet dat alle laboratoria dezelfde uitkomsten produceren (Cowgill, 1987; Baird *et al.*, 1989a). Er treedt een aanzienlijke variatie op, die mogelijk wordt veroorzaakt door verschillen in de wijze waarop de testen worden uitgevoerd en de omstandigheden waaronder de proefdieren worden gekweekt. Blijkbaar geven de richtlijnen teveel ruimte op essentiële onderdelen. Dit is niet verwonderlijk, aangezien de precieze rol van omgevingsfactoren en hun relatieve belang grotendeels onbekend zijn.

In termen van complexiteit is de afstand tussen de resultaten van een laboratoriumtest met een enkele diersoort en effecten op het niveau van een aquatisch ecosysteem enorm. Ongeveer tien jaar geleden zette Kooijman de eerste stap om deze afstand te overbruggen. Hij ontwikkelde een wiskundig model om toxische effecten op het niveau van een individuele watervlo, zoals die kunnen worden waargenomen in laboratoriumtesten, te vertalen naar effecten op populaties (Kooijman, 1986; Kooijman *et al.*, 1989). Door de resultaten van computersimulaties te vergelijken met het gedrag van reële laboratoriumpopulaties, ontstonden onderzoeksvragen omtrent enkele kritische aannamen van het model.

Het onderzoek dat in dit proefschrift wordt gepresenteerd, richt zich in de eerste plaats op de rol van voedsel in het leven van *D. magna*. De hoeveelheid voedsel is bepalend voor groei en voortplanting, maar kan tevens de gevoeligheid voor toxische stoffen beïnvloeden. Om een bijdrage te leveren aan verdere standaardisatie van toxiciteitstesten werd de invloed van voedsel op het kweken van proefdieren onderzocht. Tevens werden, met het oog op de ontwikkeling van extrapolatiemodellen, enkele relevante modelaannamen betreffende de relatie tussen voedselbeschikbaarheid en groei en reproductie van *Daphnia* experimenteel getoetst. Ten behoeve van het experimentele werk zijn twee methoden ontwikkeld, die hieronder worden besproken. De zware metalen cadmium, chroom en lood, alle aangemerkt als aandachtsstoffen door de Internationale Rijncommissie (1987), zijn in het onderzoek gebruikt als modeltoxicanten. Het onderzoek werd uitgevoerd bij het Rijksinstituut voor Integraal Zoetwaterbeheer en Afvalwaterbehandeling (RIZA) te Lelystad.

Ontwikkeling van nieuwe methoden

Lichaamslengte is een basisgegeven voor onderzoek naar de levensloop van organismen. Om de ontwikkeling van individuen te kunnen volgen, dienen ze levend gemeten te worden. Daarbij mag geen letsel worden toegebracht. Om lichaamslengte op een gemakkelijke en snelle manier te kunnen meten, is een computerprogramma ontwikkeld dat gebruik maakt van een commercieel verkrijgbaar beeldverwerkinggssysteem, genaamd TIM (*hoofdstuk 2*). Automatische lengtemeting van watervlooien is veel sneller dan de klassieke handmatige methode met een dissectiemicroscoop, doch minder nauwkeurig. De methode kan worden aanbevolen voor grote aantallen, bijvoorbeeld het bepalen van de lengteverdeling van een populatie. De monsters mogen overigens niet verontreinigd zijn met partikels van dezelfde grootte als de organismen die gemeten moeten worden. Als nauwkeurigheid belangrijk is, bijvoorbeeld voor het bepalen van een groeicurve, heeft de handmatige methode de voorkeur. In het voorliggende onderzoek is daarom vooral de microscoop gebruikt.

Om concurrentie binnen een *Daphnia* populatie te kunnen meten, is een bijzondere proefopstelling ontwikkeld die wordt beschreven in *hoofdstuk 6*. Deze opstelling bestond uit twee onderling verbonden vaten, zodat kweekmedium met algen van het ene vat in het andere kon stromen en vice versa. De waterstroom werd aangedreven door perslucht. In elk vat bevond zich een groep watervlooien van dezelfde leeftijd (cohort), die door gaas bijeen werd gehouden. Op deze wijze kon voedselconcurrentie tussen cohorten worden onderzocht, terwijl waarnemingen aan de afzonderlijke cohorten mogelijk waren. De reproduceerbaarheid van de experimentele resultaten was redelijk. Dit systeem is zeer geschikt voor onderzoek naar concurrentie tussen soorten en stadia die uiterlijk sterk op elkaar lijken, of om andere redenen niet bij elkaar gezet kunnen worden. Verschillende voedselregimes kunnen worden getest en het systeem kan eenvoudig uitgebreid worden met een of meer vaten.

Standaardisatie

De huidige (internationale) richtlijnen voor toxiciteitstesten met *D. magna* besteden voornamelijk aandacht aan het standaardiseren van de test zelf. De kweek van testorganismen wordt min of meer vrij gelaten. Een gevolg hiervan is, dat elk laboratorium zijn eigen kweekmethode hanteert. Als voorbeeld van een stabiele kweek wordt in *hoofdstuk 2* de methode van het RIZA beschreven. In het voorliggende onderzoek werden in IJsselmeerwater gekweekte *Daphnia*'s gebruikt. Als voedsel werd dagelijks een vaste hoeveelheid van de eencellige groenalg *Chlorella pyrenoidosa* toegediend. De kweekmethode wordt omschreven met de term 'batch-cultuur', wat betekent dat de dieren groepsgewijs gehuisvest zijn, waarbij een groep uit een enkel cohort bestaat. De aquaria werden driemaal per week ververst, waarbij tevens de jongen verwijderd werden. Doordat groeiende watervlooien steeds meer gaan eten en hun jongen tot maximaal drie dagen na de geboorte meeëten, treden schommelingen op in algendichtheid. Dit kan invloed hebben op de resultaten van toxiciteitstesten, daar de voedingstoestand van moederdieren van invloed is op aantal en kwaliteit van de jongen. Door Cowgill *et al.* (1985) is voor laboratoriumpopulaties van *D. magna* aangetoond dat er een omgekeerde relatie bestaat tussen de grootte van een broedsel en het gewicht van de pasgeboren jongen. Deze waarneming wees op het bestaan van een voortplantingsstrategie,

die aan de hand van veldwaarnemingen reeds eerder was voorgesteld door Hutchinson (1951). Volgens deze hypothese is de voortplanting onder gunstige omstandigheden gericht op verspreiding van de genen, door vele kleine, dus 'goedkope' jongen te produceren. In perioden van schaarste worden juist heel weinig, maar wel grote, jongen geboren, die stressbestendiger zijn. Experimentele toetsing van deze hypothese en het onderzoeken van de consequenties voor ecotoxicologie en ecologie vormen een belangrijk doel van dit proefschrift (zie ***hoofdstukken 3 en 4***).

Uit onze experimenten bleek dat watervlooien-wijfjes bij 20°C in ca. acht dagen hun voortplanting kunnen aanpassen aan een plotselinge verandering in de voedselgift. In de aanpassingsperiode vervelden de dieren driemaal. Vermeerdering of vermindering van de voedselhoeveelheid vond plaats tussen het derde en vierde broedsel. Na aanpassing varieerde de broedselgrootte van 7 tot 76 jongen en de lichaamslengte van de pasgeborenen lag tussen 0,92 en 1,1 mm. De allerkleinste jongen (0,80 mm) werden gevonden in het eerste broedsel van de proefdieren. Het laatste verschijnsel is welbekend en er wordt rekening mee gehouden in richtlijnen voor toxiciteitstesten (bijvoorbeeld OECD, 1984). Zij raden het gebruik van het eerste broedsel af in verband met een verhoogd risico voor sterfte in de blanco's, waardoor de testresultaten ongeldig kunnen worden (zie ook Cowgill *et al.*, 1986). De voorliggende studie laat ook zien dat grote jongen veel meer energiereserves van hun moeder meekrijgen dan kleine. Om deze reden kan een positieve relatie verwacht worden tussen overleving onder hongercondities en lichaamsgrootte. Dit verband is inderdaad waargenomen in hongerexperimenten, die worden beschreven in ***hoofdstuk 5*** en Enserink (1989). Vergelijkbare experimenten (zie ***hoofdstuk 4***) leverden echter een minder duidelijk resultaat op. Wat de blancosterfte in acute 48 uurs-testen zonder voedsel betreft, is het interessant om op te merken dat mediane overlevingstijden nooit korter waren dan 4 dagen, zelfs niet voor de kleinste (0,77 mm) jongen.

Een nog belangrijker reden om de grootte van pasgeboren jongen in de gaten te houden is dat kleine jongen gevoeliger kunnen zijn voor acute blootstelling aan chemicaliën dan grotere exemplaren (***hoofdstuk 3***). In proeven met cadmium was de concentratie waarbij de helft van de proefdieren sterft (LC_{50}) voor kleine jongen driemaal zo laag als voor grote jongen. Dit verschil ontbrak echter in testen met chroom, waar alle LC_{50}'s gelijk waren. Verschillende werkingsmechanismen van deze twee metalen zijn een mogelijke oorzaak van dit verschijnsel.

In chronische reproductietesten van 21 dagen met dezelfde stoffen bleek de aanvankelijke grootte van de proefdieren nauwelijks effect te hebben op hun gevoeligheid voor cadmium en chroom (***hoofdstuk 4***). Herhaalde testen leverden geen consistent beeld op, wat wel het geval was bij de acute testen (zie ook Enserink, 1989). Blijkbaar worden minieme verschillen in de aanvankelijke kwaliteit van de proefdieren in de loop van een chronische test gemaskeerd door andere factoren, zoals voedsel. Vergelijking van testen die na elkaar werden uitgevoerd suggereert dat er een klein verschil in voedselomstandigheden was. De variatie in effectconcentraties die hierdoor ontstond, was van dezelfde orde als het effect van de grootte van de proefdieren aan het begin van de test, namelijk een factor 2.

Het meest opvallende resultaat van deze studie is dat de voedingstoestand van moederdieren van wezenlijk belang is voor de kwaliteit van de jongen en daarmee voor de resultaten van acute

toxiciteitstesten (Enserink, 1989; Enserink *et al.*, 1990). Onafhankelijk van ons onderzoek zijn vergelijkbare resultaten geboekt door Baird *et al.* (1989b). Recentelijk zijn deze bevindingen door andere onderzoekers bevestigd.

De voortplantingsstrategie van *D. magna*, zoals hierboven beschreven, werd ook door Cox *et al.* (1992), Naylor *et al.* (1992) en Viganò (1993) gevonden. Een omgekeerd verband tussen de voedingstoestand van de moeder en de grootte van de jongen blijkt ook te gelden voor het drooggewicht, hoewel deze parameter variabeler is dan lichaamslengte (Naylor *et al.*, 1992). Zelfs onder kweekomstandigheden, waarbij men juist probeert om de voedselbeschikbaarheid zo constant mogelijk te houden, treden oscillaties op in broedselgrootte, die negatief gecorreleerd zijn aan lengte en gewicht van de pasgeborenen (Viganò, 1993; Lazorchak & Waller, 1993). Dergelijke fluctuaties kunnen veroorzaakt worden door veranderingen in de kwaliteit of kwantiteit van het toegediende voedsel, toename van de voedselopname door groeiende *Daphnia*'s en tijdelijke aanwezigheid van meeëtende jongen.

In onze experimenten werd de gevoeligheid van jonge *D.magna* voor acute blootstelling aan cadmium beïnvloed door de voedselvoorziening van de oudergeneratie. Deze factor had echter geen effect in testen met chroom. Blijkbaar is dit verschijnsel afhankelijk van de toetsstof. Het grootste effect van kweekomstandigheden op de acute LC_{50}, namelijk een factor 6, is tot nu toe gerapporteerd voor 3,4-dichlooraniline (Baird *et al.*, 1991). Voor 3,4-dichlooraniline, natriumbromide (Naylor *et al.*, 1992) en koper (Lazorchak & Waller, 1993) zijn eveneens significant positieve relaties tussen lichaamsgrootte en LC_{50} waargenomen. Viganò (1993) vond geen verband in testen met ethylbenzeen en *n*-butylbenzeen. Dit heeft wellicht te maken met een zeer geringe spreiding in de grootte van de proefdieren. Opmerkelijk genoeg werd het verband tussen cadmiumtoxiciteit en de voedselsituatie van de moederdieren wel bevestigd (Baird *et al.*, 1991), maar de relatie met de lengte van de proefdieren niet (Naylor *et al.*, 1992). Er zijn meerdere verschillen tussen de experimenten van Naylor en onze proeven die een verklaring kunnen bieden. Zo gaven zij geen voedsel gedurende de test, ze gebruikten verschillende klonen van *D. magna* en een ander testmedium. Hierbij dient te worden opgemerkt dat beide onderzoeken werden uitgevoerd volgens officiële, doch verschillende richtlijnen. Bedroeg het verschil tussen de hoogste en de laagste LC_{50} voor cadmium als gevolg van voedselomstandigheden in de kweek reeds een factor 3 (zie ook Baird *et al.*, 1991), een nog grotere variatie trad op tussen de laboratoria. De gemiddelde LC_{50} voor cadmium was in onze experimenten een factor 20 hoger dan in het laboratorium waar de experimenten van Naylor en Baird werden uitgevoerd. Met het oog op standaardisatie zijn bronnen van variatie binnen een laboratorium en tussen laboratoria even belangwekkend.

In het bovenstaande is duidelijk de relevantie aangetoond van de voedselvoorziening in *Daphnia* kweken voor de uitkomsten van toxiciteitstesten. Ik pleit dan ook voor uitbreiding van de huidige richtlijnen en standaardisatieprogramma's met regels voor het kweken van proefdieren. Behalve voedsel in de kweek, beïnvloeden ook andere factoren de testresultaten, zoals voedsel in de testen (Winner *et al.*, 1977; Chandini, 1988a, b; Soares, 1989; Lazorchak & Waller, 1993; Sims *et al.*, 1993; Klüttgen & Ratte, 1994), de genetische opmaak van het proefdier (Soares, 1989; Baird *et*

al., 1991), de samenstelling van het medium (Winner, 1985) en ook statistische gegevensverwerking (Hoekstra, 1993). Slechts weinig is echter bekend over het relatieve belang van deze factoren en hun interactie. De zogenaamde ringtest is een zeer geschikt instrument voor het onderzoeken van samengestelde effecten. Momenteel wordt de laatste hand gelegd aan een voorstel voor verbetering van de OECD-richtlijn voor chronische toxiciteitstesten uit 1984, op basis van de resultaten van een internationale ringtest. Dit onderzoek betrof de effecten van genotype, medium en voedsel in de test en werd gecoördineerd door de Universiteit van Sheffield (UK), Department of Animal & Plant Sciences. Om voldoende inzicht in bronnen van variatie, binnen en tussen laboratoria, te verkrijgen en om de belangrijkste factoren te identificeren is verder onderzoek noodzakelijk. Met de huidige richtlijnen als uitgangspunt, kunnen standaardtesten met *D. magna* hun naam nog niet waarmaken. Hoewel ik beslist een voorstander ben van het gebruik van *Daphnia*-testen in waterkwaliteitsbeleid en -beheer, moet ik concluderen dat de variatie in de uitkomsten van deze testen de betrouwbaarheid van afgeleide kwaliteitsdoelstellingen negatief beïnvloedt. Ik ben van mening dat een betere beheersing van testresultaten mogelijk is. Vanuit gezamenlijke afspraken over:

- de gewenste kwaliteit van testresultaten voor waterkwaliteitsbeheer en
- de hiervoor benodigde mate van detaillering van richtlijnen voor kweken en toxiciteitstesten

zou aan de verbetering van testresultaten gewerkt moeten worden. Hiermee kan een steviger basis voor waterkwaliteitsdoelstellingen en de beoordeling van milieumonsters gecreëerd worden.

Extrapolatie

Watervlooien bezitten een indrukwekkend aanpassingsvermogen als het om voedsel gaat. Zij zijn in staat om alle eigenschappen die van levensbelang zijn snel en adequaat te veranderen, als de situatie zich wijzigt. Dit vermogen is ongetwijfeld van evolutionair belang, daar de leefomgeving van *Daphnia* wordt gekenmerkt door veranderingen in het voedselaanbod. De uitdaging voor ontwerpers van *Daphnia*-modellen is om juist die eigenschappen en relaties in hun model te vangen, die van wezenlijk belang zijn voor het doel van hun model.

In het model van Kooijman (1986), dat de groei en ontwikkeling van individuele *Daphnia* beschrijft, is gestreefd naar een balans tussen wiskundige eenvoud en biologisch realisme. Wiskundig gesimuleerde individuen konden worden samengevoegd tot eveneens gesimuleerde populaties (Kooijman *et al.*, 1989; Van der Hoeven, 1991). In principe kunnen op deze wijze de effecten van chemicaliën op individuen vertaald worden naar effecten op het gedrag van populaties. Dit werd door de ontwerpers van deze modellen beschouwd als een eerste stap in de richting van het voorspellen van toxische effecten op ecosysteem-niveau. Ecosystemen bestaan uit een ingewikkeld netwerk van populaties die elkaar beïnvloeden. Om het ontstaan van een gordiaanse knoop van gedetailleerde submodellen te voorkomen, werd begonnen met een zo algemeen mogelijke verzameling van aannamen. Deze aannamen waren gebaseerd op ideeën over fysische processen, op de veronderstelling dat alles uitermate eenvoudig is totdat bewezen wordt dat het toch ingewikkelder is, en op proefondervindelijk verkregen gegevens. Het was de bedoeling om in het ontwikkelingsproces van een model voor individuen naar een ecosysteemmodel steeds meer

details te verwijderen. Het *Daphnia*-model was dan ook bedoeld als proef voor een meer algemeen model, dat toepasbaar zou moeten zijn op meerdere soorten (Kooijman *et al.*, 1987). Niettemin is een bepaalde mate van detaillering onontbeerlijk voor bruikbare extrapolaties naar hogere organisatieniveaus.

Een overzichtelijke opsomming van Kooijman's (1986) modelaannamen wordt gegeven door van der Hoeven (1991). In het voorliggende proefschrift wordt op basis van experimentele resultaten een aantal van deze aannamen geëvalueerd:

- de grootte van pasgeboren *Daphnia*'s is constant;
- de grootte-gerelateerde energievoorraad van het pasgeboren jong is gelijk aan die van de moeder;
- voortplanting kan alleen plaatsvinden boven een bepaalde lichaamslengte;
- een vast gedeelte van de beschikbare energie wordt besteed aan voortplanting en het resterende deel is beschikbaar voor groei en onderhoud van het lichaam;
- de opnamesnelheid van voedsel is evenredig met het lichaamsoppervlak;
- de energiekosten voor lichaamsonderhoud zijn evenredig met het gewicht.

Tevens is een poging gedaan om het belang van deze relaties voor extrapolatiedoeleinden aan te geven.

In ***hoofdstuk 3 en 4*** wordt de voortplantingsstrategie van *D. magna* in relatie tot voedselbeschikbaarheid beschreven. Lichaamsgrootte en energievoorraad van pasgeboren jongen waren negatief gecorreleerd met de voedingstoestand van hun moeder. Volgens Cox *et al.* (1992) neemt de grootte van de jongen niet verder af boven een bepaald verzadigingsniveau voor voedsel. Deze waarnemingen conflicteren met twee modelaannamen, namelijk dat de grootte van pasgeborenen onafhankelijk is van de voedselomstandigheden en dat hun relatieve energie-inhoud identiek is aan die van hun moeder. De hierboven geschetste voortplantingsstrategie is van groot belang voor de overleving en ontwikkeling van jonge *Daphnia*'s bij voedselschaarste. Bij afwezigheid van voedsel kan de overlevingstijd toenemen van 4 tot 9 dagen met toenemende lichaamsgrootte (***hoofdstuk 5***). Bovendien kunnen grote, vette jongen doorgroeien tot in het derde vervellingsstadium (***hoofdstuk 4***). Hierdoor kan de periode tot het eerste broedsel bekort worden, zoals hieronder zal worden aangetoond.

In het *Daphnia*-model worden de eerste eieren in de broedzak gedeponeerd als het wijfje een bepaalde, voedselonafhankelijke lichaamsgrootte heeft bereikt. Uit ***hoofdstuk 5*** blijkt echter dat voedsel een duidelijke en significante invloed heeft op de lichaamsgrootte als de eerste eieren gelegd worden. De kleinste moederdieren werden waargenomen bij tussenliggende voedselcondities. Een duidelijker grenswaarde voor de start van de eiproduktie werd twee stadia eerder gevonden, wat in overeenstemming is met de resultaten van Ebert (1992). Deze minimumgrootte was echter niet volledig onafhankelijk van voedsel. Bij een betrekkelijk lage voedselbeschikbaarheid kreeg groei soms een hogere prioriteit dan voortplanting. Het eerste broedsel werd in deze gevallen veel later gelegd en het bestond uit grotere jongen dan bij de dieren die zich, onder dezelfde omstandigheden, vroeg voortplantten. Mogelijk bestaat er een verband

tussen toenemende duur van een vervellingsstadium en grotere jongen. Geconcludeerd wordt dat deze voedselhoeveelheid precies voldoende energie opleverde om een enkel ei te produceren.

Een opmerkelijk verschijnsel werd waargenomen bij voedselschaarste. Transport van vetreserves naar de ovaria, waar het wordt omgezet in dooier, bleek omkeerbaar te zijn. Ophoping in het ovarium gedurende de eerste helft van een vervellingsstadium en het opnieuw verdwijnen van dooier tegen het einde van een stadium zijn sterke aanwijzingen dat de aanmaak van een nieuwe carapax (schild) een hogere prioriteit heeft dan de produktie van eieren. De huidige *Daphnia*-modellen houden overigens geen rekening met de mogelijkheid van herverdeling van energiereserves die bestemd zijn voor de eiproduktie (Hanstveit *et al.*, 1987; Gurney *et al.*, 1990).

Uit de verdeling van biomassa gedurende de produktie van het eerste broedsel bleek dat de investering in voortplanting verhoudingsgewijs toenam met de hoeveelheid voedsel. Hetzelfde is waargenomen door McCauley *et al.* (1990). Ook in *Daphnia*-modellen wordt een deel van de beschikbare energie gereserveerd voor de produktie van eieren. In het model van Kooijman wordt echter uitgegaan van een constant percentage, dat alleen onder hongeromstandigheden voor het eigen lichaamsonderhoud kan worden bestemd. Gurney *et al.* (1990) verbeterden de resultaten van hun *Daphnia*-model door dit percentage voedselafhankelijk te maken.

In ***hoofdstuk 5*** werden gecombineerde effecten van voedsel en lood op de start van de voortplanting onderzocht. Een significante interactie tussen deze factoren werd gevonden, namelijk de effecten van lood veranderden met de voedselbeschikbaarheid. Zowel bij veel als bij weinig voedsel nam de lichaamsgroei af onder invloed van lood. In het eerste geval had dit tot gevolg dat de *Daphnia*'s kleiner waren op het moment dat ze de eerste eieren legden en ook hun jongen waren kleiner. Bij weinig voedsel werd de produktie van het eerste broedsel uitgesteld en stierf bovendien een deel van de eieren en embryo's. Uitgezonderd de sterfte van het broedsel, heeft lood eenzelfde effect als vermindering van het voedselaanbod. Vergelijkbare waarnemingen zijn gedaan in testen met koper (Winner et el., 1977) en cadmium (Chandini, 1989; Klüttgen & Ratte, 1994). Aangezien in chronische toxiciteitstesten meestal een aanzienlijke hoeveelheid voedsel wordt toegediend, kan extrapolatie naar veldomstandigheden, waar vaak schaarste heerst, tot verkeerde conclusies leiden.

Laboratoriumpopulaties die een constante hoeveelheid voedsel krijgen toegediend, vertonen vaak oscillaties in het totaal aantal individuen. Het is echter onduidelijk in hoeverre dit verschijnsel een gevolg is van eigenschappen van de populaties zelf, of van onregelmatigheden in de uitvoering van het experiment. Na analyse van gepubliceerde gegevens over de dynamiek van laboratoriumpopulaties concludeerde van der Hoeven (1989) dat de laatste optie zeer wel mogelijk is. De populatiesimulaties op basis van Kooijman's *Daphnia*-model, waarin zeer sterke en aanhoudende oscillaties het beeld bepaalden, werden dan ook in twijfel getrokken (Kooijman *et al.*, 1989). De belangrijkste drijvende kracht achter deze oscillaties is synchronisatie van de ontwikkeling van de individuele *Daphnia*'s. Deze synchronisatie treedt op wanneer de populatiedichtheid afneemt als gevolg van een enorme concurrentiestrijd om een beperkte hoeveelheid voedsel. Aan de basis van de voorspelde synchronisatie liggen twee modelaannamen, namelijk dat voedselopname evenredig is met de oppervlakte (lengte^2) en onderhoudskosten evenredig met het gewicht (lengte^3) van de watervlo. Dit heeft tot gevolg dat de groeisnelheid

afneemt naarmate het dier groter wordt en haar maximale lichaamsgrootte nadert. Deze maximale grootte is bij lagere voedseldichtheden overigens geringer dan bij veel voedsel. Dieren die kleiner zijn dan het maximum kunnen nog doorgroeien, terwijl de groei van dieren boven het maximum stagneert. Dit mechanisme leidt tot convergentie van lichaamslengten. Het bovengeschetste groeimodel is genoemd naar von Bertalanffy (1969, type I). In de simulaties van Kooijman wordt ook de voortplanting gesynchroniseerd, doordat vele individuen tegelijk de minimale lengte voor het leggen van de eerste eieren bereiken (zie boven).

Het proces van convergentie en synchronisatie is experimenteel onderzocht (*hoofdstuk 6*). Hiertoe werden groei en ontwikkeling van twee cohorten van jonge *D. magna* gevolgd onder proefomstandigheden die zoveel mogelijk leken op de situatie tijdens het dichtheidsmaximum van een laboratoriumpopulatie en de daaropvolgende afname (zie boven). De cohorten, die bestonden uit twee opeenvolgende broedsels van een gezamenlijk moedercohort, waren ruimtelijk gescheiden, terwijl ze hetzelfde voedsel en medium deelden. Om het effect van fluctuaties in voedseldichtheid te onderzoeken werden twee regimes ingesteld: constante toediening of eenmaal daags. Onder deze proefomstandigheden was convergentie van lichaamslengten afwezig of heel traag. Van synchronisatie van het eerste broedsel was geen sprake. Bij een constante, zeer lage voedselconcentratie was het oudere cohort, dat uit grotere dieren bestond, de sterkste concurrent, terwijl fluctuerende voedselconcentraties meer in het voordeel waren van het jongere cohort. De convergentiesnelheden die op basis van het von Bertalanffy model werden berekend waren veel te hoog. De geschiktheid van dit model voor het beschrijven van de processen onder deze experimentele omstandigheden, is dan ook vergeleken met andere groeimodellen. De waargenomen, min of meer parallele groei van de cohorten kan beschreven worden met een lineair groeimodel voor dieren die nog niet volwassen zijn (juvenielen). Hierbij dient opgemerkt te worden dat de vorm van een groeicurve sterk afhankelijk is van de (proef-)omstandigheden.

Op basis van de hierboven beschreven mechanismen concludeer ik dat sterke synchronisatie van individuele groei en ontwikkeling en heftige oscillaties in de populatiedichtheid onwaarschijnlijk zijn. Parallele groeicurven, een toename in individuele variatie bij lage voedselconcentraties (zie ook Cox *et al.*, 1992) en verschuivingen in het gebruik van energie voor groei of eiproduktie hebben juist een stabiliserende werking. Daarnaast is een omgekeerd verband aangetoond tussen de energievoorraad van pasgeboren jongen en die van de moeder. Hierdoor zal bij lage voedselconcentraties de groeisnelheid van de populatie afnemen, omdat van een bepaalde hoeveelheid energie minder jongen worden gemaakt dan verondersteld wordt in het model. Bovendien zal de overlevingskans van deze jongen toenemen. In het algemeen kan worden gesteld dat het aanpassingsvermogen van *D. magna*, in het bijzonder aan voedselschaarste, door de huidige modelaannamen wordt onderschat. Eenvoudige modellen kunnen goed bruikbaar zijn voor het simuleren van een individuele *Daphnia*. Het is de vraag of ze ook een acceptabele beschrijving geven van de dynamiek van een populatie. Toepassing van meer biologische informatie zal een gunstig effect hebben op de modelsimulaties en daarmee op de vertaling van toxische effecten op individuen naar effecten op populatieniveau. De balans tussen elegante wiskundige formuleringen en de complexe biologie van een watervlo is echter zeer gevoelig. Daarom zal een stapsgewijze toepassing van de belangrijkste biologische mechanismen hand in hand moeten gaan met

gevoeligheidsanalyses van het model. Dit proces zal ons inzicht in het gedrag van populaties naar verwachting doen toenemen.

De resultaten van *hoofdstuk 5* en de negatieve effecten van vele toxicanten op groei en voortplanting (zie bijvoorbeeld *hoofdstuk 4* en Enserink *et al.*, 1991) vormen een sterke aanwijzing dat toxische stoffen de zeer efficiënte benutting van voedsel door watervlooien verstoren. Hierdoor zouden eetbare algen over kunnen blijven, wat verhoogde algendichtheden tot gevolg heeft. Effecten van toxicanten op ecosystemen zijn evenwel afhankelijk van vele factoren, waaronder de relatieve gevoeligheden van soorten en functionele groepen, zoals algen, grazers en roofdieren. Marshall & Mellinger (1980) observeerden bijvoorbeeld zowel afname als toename van de algenproduktie in zakkenproeven met planktongemeenschappen, die aan cadmium werden blootgesteld. Bij afname werd een direct effect op de primaire produktie gevonden en bij toename werden toxische effecten op algen overgecompenseerd door verlaging van de zoöplanktondichtheid. Een algemene conclusie van mesocosmosonderzoek met bestrijdingsmiddelen is dat primaire effecten op veldpopulaties redelijk goed kunnen worden voorspeld, op basis van laboratoriumtesten met dezelfde soorten organismen. De blootstelling aan de stof moet dan wel bekend zijn. Meestal is het echter niet mogelijk om secundaire effecten te voorspellen (Brock & Budde, 1994). Onlangs stelden Scholten *et al.* (1994) dat de huidige eutrofiëringsproblemen in oppervlaktewater vooral veroorzaakt worden door toxische stoffen, die de graascapaciteit van het herbivore zoöplankton aantasten. Hoewel in Nederland veel, deels terechte kritiek op dit onderzoek werd geuit, denk ik dat bovenstaande stelling een waardevolle hypothese voor toekomstige studies is. De rol van toxische stoffen in aquatische ecosystem is nog grotendeels onbekend. Onderzoek naar de gecombineerde effecten van nutriënten en microverontreinigingen kan onze kennis op essentiële onderdelen vergroten. Bovendien brengt het ecologen en ecotoxicologen nader tot elkaar.

Curriculum vitae

Lisette Enserink werd geboren op 2 februari 1961 in Amsterdam. In 1979 behaalde zij het diploma ongedeeld VWO aan de scholengemeenschap Snellius in Amstelveen. In hetzelfde jaar begon zij de studie biologie aan de Rijksuniversiteit Utrecht. De doctoraalfase omvatte de vakken maatschappelijke biologie (RUU), zoölogische ecologie en taxonomie (NIOZ), scheikundige dierfysiologie (RUU) en biologische toxicologie (IOB), en de nevenrichting informatica. Tijdens haar studie was zij als vrijwilligster verbonden aan de Biologiewinkel. In augustus 1986 behaalde zij het doctoraaldiploma.

Van 1 december 1986 tot 1 december 1991 werkte zij bij het RIZA als AIO/wetenschappelijk assistent, afwisselend in dienst van het RIZA en de vakgroep Toxicologie van de Landbouwuniversiteit Wageningen. Het onderzoek waar dit proefschrift over handelt werd gedurende deze periode verricht.

Op 1 december 1991 werd zij aangesteld bij de hoofdafdeling Water van de directie Noord-Holland van Rijkswaterstaat. In haar huidige functie initieert en begeleidt zij projekten op het gebied van natuurontwikkeling en ecologisch herstel, alsmede diffuse verontreiniging van watersystemen. Daarnaast werkt zij aan het concretiseren van integraal waterbeheer, met name voor de Waddenzee.

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Lisette Enserink
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