# THE EFFECTS OF SELECTED REFERENCE TOXICANTS ON EMBRYONIC DEVELOPMENT OF THE FRESHWATER SHRIMP *CARIDINA NILOTICA* (DECAPODA: ATYIDAE)

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This thesis is submitted in fulfillment of the requirements for the degree of Master of Science at Rhodes University.

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## ABSTRACT

Aquatic toxicity tests are increasingly being used in water resource management worldwide, and currently in South Africa, policy and legislation are being drafted to reflect this international trend. While standard toxicity test methods and test organisms are being considered to develop and set water quality guidelines and effluent discharge limits, it is not clear whether guidelines and discharge limits set using these standard test organisms will be sufficient to protect South Africa's scarce water resources. As part of on-going research to investigate the use of indigenous riverine organisms as toxicity test organisms a number of potential species have been identified, including the freshwater shrimp *Caridina nilotica*.

For much of the history of aquatic toxicological data the bulk of the data has been generated by acute toxicity testing, based on short exposures and using mortality as the response end point. There are relatively few chronic, long-term tests with sub-lethal endpoints. However, it was recognized that information about longer exposure durations and non lethal response endpoints was needed, instead of mortality. Chronic tests can provide a more environmentally realistic measure of chemical toxicity than acute toxicity tests.

*Caridina nilotica* has been identified as a potential standard toxicity test organism, as it is widely distributed, easy to find and it occurs in flowing waters. It is an indigenous species which can be easily cultured and maintained in the laboratory and is also ecologically important. Both adults and juveniles have been used successfully in acute toxicity tests at the Institute for Water Research (Rhodes University) and the ability to rear the organisms under laboratory conditions has allowed the development of chronic toxicity tests using *C. nilotica*.

Chronic early life stage tests include continuous exposure of the early life stages, which are presumed to be the most sensitive for aquatic organisms. This study reports on the embryonic development of *C. nilotica* at the culture temperature of $24^0$C. Morphological developmental stages were monitored

and measured and 7 developmental stages were identified. Based on the measurements of the features that were identified, toxicity tests using the reference chemicals sodium chloride (NaCl), sodium sulphate ($Na_2SO_4$) and cadmium chloride ($CdCl_2$) were undertaken to test the suitability of *C. nilotica* embryonic development for chronic toxicity tests for use in water resource management. The length, width, length:width ratios and area of the features decreased in size when exposed to the chemicals. The Lowest Observed Effect Concentration (LOEC) values were 2000mg/L for $Na_2SO_4$, 3000mg/L for NaCl and 0.31mg/L for $CdCl_2$. The No Observed Effect Concentration (NOEC) values were 1000mg/L for $Na_2SO_4$, 2000mg/L for NaCl and <0.31mg/L for $CdCl_2$. Further research on the teratogenic effects of single chemicals and industrial effluent on developing *C. nilotica* embryos needs to be undertaken in order to evaluate the described test protocol for use in water resource management.

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## DECLARATION

The following thesis has not been submitted to an university other than Rhodes University, Grahamstown, South Africa. The work presented here is that of the author.

# CHAPTER 1

## 1.1 General Introduction

Freshwater is essential for human survival and for the survival of the planet's plants and animals (Davies and Day, 1998). Freshwater ecosystems form the resource base on which water users, such as the agricultural, recreational, domestic and industrial sectors depend. These essential freshwater resources therefore need to be protected and maintained in a healthy state (Roux *et al.*, 1996). There is increasing understanding nationally and internationally that water resources can be successfully managed only if the natural, social, economic and political environments in which water occurs and is used are taken into full consideration (Roux *et al.,* 1996)**.**

Pollution, climate change, water-related diseases, and the destruction of the natural world all threaten the purity and availability of our most precious resource (Davies and Day, 1998). Natural fresh waters (i.e., ground water and surface waters) are the ultimate recipients of most toxic substances generated by industrial, agricultural, and domestic activities and released into the environment (Anderson and D'Apollonia, 1978, in Rand, 1995).

## 1.2 Water Quality Management in South Africa

South Africa is located in a predominantly semi-arid part of the world (Department of Water Affairs and Forestry, 2004). Freshwater resources are very limited and are under threat from growth in population, agricultural and industrial demand for water (Davies and Day, 1998). South Africa depends mainly on surface water resources for most of its urban, industrial and irrigation requirements. In the northern parts of the country both the surface and groundwater resources are nearly fully developed and utilized (Department of Water Affairs and Forestry, 2004). Rivers are the main sources of freshwater in South Africa, and are also the systems most under threat from water abstraction, regulation and pollution (Dallas *et al.*, 1994, Davies and Day, 1998, Palmer *et al.*,

2004). Human activities affect both the quantity and the quality of water in aquatic ecosystems (Dallas *et al.,* 1994). Rivers and other water resources are used to dispose of waste (Davies and Day, 1998).

The factors influencing water quality can either be natural or result from human activity (Palmer *et al*., 2004). Toxicants enter these aquatic systems from non-point and point sources. Point sources the readily identifiable inputs where waste is discharged to the receiving waters from a pipe or drain. Most industrial wastes are discharged to rivers and the sea in this way. Non-point sources of pollution refer to those inputs which occur over a wide area and are associated with particular land uses (e.g. agriculture), as opposed to individual point source discharges (Rand, 1995; Davies and Day, 1998).

Non-point source pollution is often more difficult to control than point source pollution. In urban areas the provision of reticulated sewerage systems and adequate street cleaning are important measures in controlling pollution, while in farming and forestry areas, soil conservation practices and the controlled application of pesticides and fertilisers are necessary if pollution of waterways is to be avoided (USEPA, 1994). Water quality refers to the physical, chemical and biological characteristics of water, and describes how suitable the water is for its intended purpose in nature or for use by different water users (Palmer *et al.*, 2004). Different ecosystems and different user groups can have widely variable water quality requirements (Palmer *et al.,* 2004). The critical issue, then, is to determine acceptable levels of any pollutant for each river (Dallas, *et al*., 1994).

### 1.2.1 National Water Policy

The Constitution of the Republic of South Africa (Act 108 of 1996) contains the Bill of Rights which gives all South Africans the right to an environment that is not harmful to their health or well being, as well as the right to have the environment protected for the benefit of present and future generations. It is therefore the duty of the Government to make sure that water pollution is prevented, that there is sufficient water to maintain the ecological integrity of water resources, and that

water conservation and sustainable, justifiable economic and social development are promoted (DWAF, 2004).

In South Africa water is regarded as a social, environmental and economic good and it is this which guides the water policy. The National Water Policy (NWP) (Department of Water Affairs and Forestry, 1997) has three fundamental objectives for managing South Africa's water resources:

- To achieve equitable access to water, that is, equity of access to water services, to the use of water resources, and to the benefits from the use of water resources.
- To achieve sustainable use of water by making progressive adjustments to water use with the objective of striking a balance between water availability and legitimate water requirements, and by implementing measures to protect water resources.
- To achieve efficient and effective water use for optimum social and economic benefit.

The objective of managing the quantity, quality and reliability of the nation's water resources is to achieve optimum, long-term, environmentally sustainable social and economic benefit for society from their use. The intention of environmentally sustainable water use is to balance water use with the protection of the resource in such a way that the resources are not degraded beyond recovery. The National Water Act (NWA) and Policy (NWP) support management of the nation's water resources (DWAF, 1997; NWA, 1998).

The national water resource strategy for the implementations of the NWP and NWA provides the framework for the protection, use, development, conservation, management and control of water resources for the country as a whole (DWAF, 2004). It also provides the framework within which water will be managed at regional or catchment level, in defined water management areas (DWAF, 2002).

### 1.2.2 National Water Act

The purpose of the National Water Act (No. 36 of 1998) (NWA, 1998) is to ensure that the nation's water resources are protected, used, developed, conserved, managed and controlled in ways which take into account meeting the basic needs of human and aquatic ecosystems of present and future generations and promoting equitable access to water (NWA, 1998). Some of the water is referred to as the Reserve, and considers both the quantity and quality of water in the resource, and will vary depending on the level of protection for the resource. The ecological Reserve relates to the water required to protect the aquatic ecosystems of the water resource. The Minister is required to determine the Reserve for all, or part of any significant water resource (NWA, 1998).

The NWA derives directly from the Fundamental Principles and Objectives for a New South African Water Law and the National Water Policy's proposals for managing water resources (NWA, 1998). Since water is essential for all life and human endeavours there are many other policies and laws, administered by a number of departments in all spheres of government, which govern activities dependent on water or affect water resources. The 1994 Water Supply and Sanitation Policy White Paper and the Water Services Act, 1997, which deal with the provision of potable water and sanitation services, are particularly closely related to the NWA (NWA, 1998).

Successful water resources management will therefore depend on co-operation among all spheres of government, and the active involvement of water users and other organisations and stakeholders (NWA, 1998). Public trusteeship does not mean that government owns the water, since the preamble to the Act recognises that "water is a natural resource that belongs to all people," but it does mean that the Minister has overall responsibility and, importantly, the authority to ensure that all water everywhere in the country is managed for the benefit of the environment and all people (NWA, 1998). This responsibility includes ensuring that water is allocated equitably, and that environmental values are promoted

(Department of Water Affairs and Forestry, 2004), therefore a tool for water quality management is required.

### 1.3 Tools for water quality management

A number of tools are available to assess water quality for ecosystem health, and it has been proposed that the combined use of assessing water physico-chemical data, biomonitoring data and ecotoxicology data be termed the environmental water quality (EWQ) approach (Palmer *et al.*, 2004). It has been suggested that this EWQ approach can be used to make water resource management decisions (Palmer *et al.*, 2004).

Managing water quality requires attention to both water resource protection and water resource use, and requires decisions about balancing the two (Palmer *et al.*, 2004). It is important to undertake water resource protection both by setting goals and objectives for water resources in the environment (resource directed measures – RDM) and by controlling water resource use activities (source directed controls – SDC). RDM provide descriptive and quantitative goals for the state of the resource, while SDC specify the criteria for controlling impacts such as waste discharge licences and abstraction licences (Palmer *et al.*, 2004).

There are three kinds of information and data that contribute to an integrated picture in the EWQ approach:

- information about the physico-chemistry of the water – gained through a chemical and physical analysis of the water;
- information about the presence, absence and abundance of biota in the ecosystem – gained through biomonitoring;
- information about the responses of specific biota to specific concentrations of chemicals or mixtures – gained through ecotoxicology (Palmer *et al.*, 2004).

In water quality monitoring, the chemical composition of effluent streams and receiving waters is measured and analysed on a regular basis. There are

drawbacks to the method. For example, because of sampling intervals, it is difficult to accurately model concentration-duration. In addition,the patchy distribution of monitoring sites, differing periods of data collection, and limited range of variables analysed, all mean that the physico-chemical data is at best representative and is always incomplete. This is a major reason for adding biomonitoring and ecotoxicology to gain an understanding of effects of pollution on aquatic ecosystems (Palmer *et al*., 2004).

Organisms respond to the whole range of stressors, not only water chemistry – so responses of organisms to stressors are not always easy to interpret. But biomonitoring has become an accepted way of measuring overall ecosystem health (Wright *et al.*, 2000). Biomonitoring offers crucial evidence of ecosystem health response (Palmer *et al*., 2004). It shows whether the community composition has changed, and whether the altered community composition comprises tougher organisms (Palmer *et al.*, 2004).

### 1.3.1 Aquatic ecotoxicology

Toxicology is the study of the effects of chemical solutions and mixtures on living organisms (Rand, 1995). Selected organisms, or communities of organisms, are exposed to single substance solutions or complex mixtures under controlled experimental conditions. The concentrations are carefully controlled and responses are reported as statistical probabilities (Palmer *et al*., 2004) and the information is used to set water quality guidelines.

Ecotoxicology is concerned with the effects of chemical and physical agents on all living systems (Rand, 1995). Environmental toxicology deals with the potential impact of chemicals, present as pollutants in the environment, to living organisms (Katzung, 1998). In assessing chemically induced effects (responses), it is important to consider that in the natural aquatic environment organisms may be exposed not only to a single chemical but to a myriad or mixture of different substances at the same or nearly the same time, which can lead to toxicological interactions (Rand, 1995).

Aquatic toxicology studies the effects of chemicals and other anthropogenic and natural material on aquatic organisms at various levels of organization. The various levels of biological organization are number of organisms killed (or surviving), reproductive success , whole-body (length and weight) or organ condition factors, number of teratogenic abnormalities or incidence of tumours, induction or inhibition of enzyme activity, and number and abundance of species in an ecological community (Rand, 1995). Effects can cause both positive and negative deviations from previously existing circumstances (Rand, 1995).

Toxicology can be used to identify chemicals that impair water quality and are detrimental to the environment and aquatic life, and can also be used to predict chemical concentrations that are either safe or concentrations at which negative impacts occur (Rand, 1995). By complementing the chemical monitoring with biomonitoring and toxicology the information that is produced can be used for water quality monitoring (Palmer *et al.*, 2004).

#### 1.3.1.1 Toxicity testing

Biological toxicity testing has become an increasingly important approach in assessing potential effects on aquatic biota, as chemical or physical tests alone are not sufficient to ensure resource protection (APHA, 1992). However, it is still very important to conduct physical or chemical monitoring in conjunction with biomonitoring and toxicity testing to determine what the organisms are being exposed to and their concentrations (Rand, 1995). This forms the fundamentals of the EWQ approach.

One of the goals of toxicological investigations is to know the relative toxicity of the substance with a view to determining its position among other toxic substances whose toxicities are already known so that the quality of the water can be improved if necessary (Rand, 1995). Of the many factors that determine whether a chemical is potentially harmful, two important considerations are the

concentration (quantity) of the chemical to which an organism is exposed and the duration of exposure to the chemical (Rand, 1995).

Toxic effects in the laboratory and the field may include both lethality (mortality) and sub-lethal effects (Rand, 1995). There are two commonly used types of toxicity tests, namely acute toxicity tests and chronic toxicity tests (Rand, 1995). Acute tests are designed to evaluate the relative toxicity of a chemical to selected aquatic organisms upon short term exposure to various, usually high, concentrations of the test chemical. The measured response effect is usually mortality. In acute exposures, organisms come in contact with the chemical delivered either in a single event or in multiple events that occur within a short period of time (Rand, 1995).

Chronic toxicity is measured in terms of sub-lethal effects such as reduced growth, reduced reproduction etc. in addition to lethality (Rand, 1995; Slabbert, 1998; USEPA, 1994). The endpoints derived from chronic toxicity response measures are usually the no observed effect concentration (NOEC), the lowest observed effect concentration (LOEC) and the effect concentration (EC) (which is also used in acute tests), based on results of the toxicity tests (Rand, 1995, USEPA, 1994). During chronic exposure in the laboratory, organisms are exposed to low concentrations of a chemical delivered either continuously or at some periodic frequency over extended periods of time (weeks, months or years), measured in relation to the organism's life cycle starting from an egg to adult (Rand, 1995).

For much of the history of aquatic toxicology, the bulk of data produced has been generated by acute toxicity testing, based on short exposures and using mortality as the response end point. There are relatively few chronic, long-term tests with sub-lethal endpoints as these are more difficult to undertake and are more expensive (Cooney, 1995). In addition, results from mesocosm-type chronic experiments are often very difficult to interpret (US EPA, 1994). However, it was recognized that information about longer exposure durations and non-lethal

response endpoints was needed, rather than only mortality data (Rand, 1995). This is because chronic toxicity tests can provide an environmentally more realistic measure of chemical toxicity than acute toxicity tests (Rand, 1995). The effective protection of aquatic populations from the potential impact of contaminants requires consideration of effects on individual species communities' survival, growth and reproduction, since these are fundamental in determining the fitness of populations in natural ecosystems (Hutchinson *et al.*, 1998).

Early lifestage tests include continuous exposure of the early life stages i.e. egg, embryo, larva, and fry of aquatic organisms to various concentrations of a chemical for the entire early life stage (Rand, 1995). Partial lifecycle tests include young juveniles and early adults (Rand, 1995). The length of exposure depends on the species (Rand, 1995). Although these tests do not provide total life cycle exposure, and therefore lack a full assessment of reproduction and other measurable endpoints as well, they do include exposure during presumed sensitive life stages (Rand, 1995). By extending the early life stage tests to include one or more complete life cycles, and observing more subtle effects of toxicants, such as changes in growth and reproduction, more accurate, direct, estimates of the threshold or safe concentration of the toxicant could be obtained (USEPA, 1994). Since the first chronic toxicity tests with fish were conducted, debate has continued regarding whether certain life stages of aquatic organisms are particularly sensitive to chemical toxicants (USEPA, 1994).

Hutchinson *et al*. (1998) had undertaken a number of analyses in a preliminary attempt to quantify the relationship of toxicant sensitivity to life stage in aquatic organisms. The emphasis of this approach focussed on data available within the Ecetoc Aquatic Toxicity (EAT) database for the key taxa used in the current European Community's aquatic environmental risk assessment procedures, namely fish and invertebrates. The observation was that the immature stages of several freshwater invertebrates were more sensitive than the older stages. However, further testing of this observation is required.

#### 1.3.1.2 Toxicity test organisms

The development of scientifically sound protocols for toxicity testing is an extensive process (USEPA, 1994). Not only does it require the identification of suitable candidate organisms, but also the establishment of appropriate and reproducible techniques with which to assess the sensitivity of these organisms to a range of chemicals (Rand, 1995). To be of practical use in such toxicity tests, a candidate species, or at least one of its life history stages, should not only be sensitive to potential contaminants, but should also be relatively easy to collect from the field (*i.e.* be abundant) as well as amenable to routine maintenance, culture and rearing in the laboratory (Rand, 1995).

In order to extrapolate meaningful, relevant and ecologically significant results from aquatic toxicity tests for the management of resource water quality, several factors should be considered in selecting appropriate test organisms:

- it must be sensitive
- it must be widely available and abundant
- it should be an indigenous species to the ecosystem that may receive the impact
- species must be ecologically important
- it should be able to be maintained and cultured in the laboratory
- and there should also be adequate background information on the species (Rand, 1995).

Protocols for standard toxicity tests in South Africa are based on internationally accepted protocols that are dominated by non-indigenous test organisms such as daphnids (e.g. *Daphnia pulex*), guppies (*Poecillia reticulata*) and fathead minnows (*Pimephales promelas*) (Cooney, 1995). As a result, few protocols exist for using indigenous South African species in toxicity tests (Department of Water Affairs and Forestry, 2000) and hence, South African water quality guidelines are currently heavily weighted by international toxicological response data.

The Unilever Centre for Environmental Water Quality (UCEWQ) at the Institute for Water Research (IWR) (Rhodes University, Grahamstown) has a database of acute and chronic toxicity data for South African aquatic macroinvertebrates (Palmer *et al*., 2004), for limited toxicants. Tolerance data for indigenous species such as *Caridina nilotica* will provide important data for the existing data and for the refinement of South African Water Quality Guidelines for aquatic resources.

### 1.4 *Caridina nilotica* as a potential standard toxicity test organism

The Atyidae is a major family of largely freshwater decapod shrimps (Hart, 1980). The atyids are widespread and frequently abundant in fresh waters of the tropics, although some extend into cooler regions (Hart, 1983). According to Barnard (1950) quoted by Hart (1983), the distribution of *Caridina nilotica* in South Africa extends as far south as the Umzimvubu river and as far west as Lake Ngami. Hart (1983) stated that specimens of *C. nilotica* had been collected in the Keiskamma and Bushmans Rivers (Eastern Cape), representing a southerly extension of its distribution. In 1984, a further southerly extension to *C. nilotica* distribution was determined when specimens were found in the Gamtoos River (Coetzee, 1989). Hart (1983) found that *C. nilotica* have a temperature activity range of 11.5 to 31.5 °C and concluded that temperature tolerance may be a significant factor affecting this species'` distribution. Scattered observations suggest that *C. nilotica* is widespread elsewhere in warm waters at least in the eastern Central and North Africa (Hart, 1981).

Species within the genus *Caridina* are recognised as being of considerable ecological importance (Hart, 1980, 1981, 1983). The genus *Caridina* performs an ecological role in the food web as herbivorous-detritivorous surface scrapers and scavengers. The feeding activities of *C. nilotica* plays a role in the clearing of debris and epiphytic microflora from the leaves of submerged macrophytes, thereby enhancing macrophyte photosynthesis and recycling organic matter (Begg & Junor, 1971 in Hart, 1981, Hussein and Obuid-Allah, 1992). *Caridina nilotica* is an important representative of the genus in tropical and subtropical Africa and is an important component of the communities of submerged

macrophyte beds and of the profundal benthos (Hart, 1980, 1981). Considering the wide distribution and abundance in suitable habitats (Hart, 1983), surprisingly little is known of the ecology of *C. nilotica* with Hart *et al.* (2003) being the most recent published research. The morphological characteristics of this genus are indicative of a slow-flowing or standing-water group (Hart, 1981). *Caridina nilotica* occurs in lentic and lotic waters in southern Africa (Hart, 1981, Hart *et al*., 2003).

In Lake Sibaya, *Caridina nilotica* dominates the littoral benthos but it is not a major dietary item of cichlids or of the catfish *Clarias gariepinus.* In Lake Kariba, this shrimp has been recorded in the diet of seven fish species (Begg and Junor, 1971 in Hart, 1981) and is eaten by a variety of birds, mainly herons (Begg, 1973 in Hart, 1981). The *Caridina* genus has a highly complicated and specialized feeding mechanism; and is considered a microphagous chelate raptatory feeder (Day *et al.*, 2001; Hart *et al.*, 2001). *Caridina* feeds principally on periphyton scraped from aquatic hydrophytes and on plant detritus, but readily scavenges on the corpses of animals such as fish, insects, and shrimps (Day *et al.*, 2001; Hart *et al.*, 2001).

The atyids also collect diatoms which they eventually release with faeces where the diatoms are presumably allowed to regenerate their nutritious coats, thus eventually providing another meal of diatoms upon which shrimp can feed (Schram, 1986). *Caridina nilotica* uses its highly specialized chelae to scrape the surfaces of organic and inorganic substrates. In Lake Sibaya and Lake Victoria, two of the few places where ecological studies of the species have been undertaken, the diet of *C. nilotica* consists largely of auto- and hetero-trophic microbiota (Hart, 1981, Hart et al., 2003).

*Caridina* reproduce sexually and are protandrous hermaphrodites (Schram, 1986). Hart (1981) and Day *et al*. (2001) stated that *C. nilotica* breed all year round with no resting stage evident in the life cycle. The success of reproduction in most crustaceans may be due to the fact that the female carries the eggs in a brood pouch with yolk, with no further nutrient supply from the parent, and palpates and turns the eggs over regularly for aeration until hatching (Adiyodi

and Adiyodi, 1994; Calow, 1981 and Schram, 1986). This may result in a high survival rate of the eggs (Hart *et al.*, 2001; Landau and Laufer, 1999; Schram, 1986). Protection and ventilation are considered two of the most important functions of crustacean maternal care (Adiyodi and Adiyodi, 1994). According to Hart (1980) and Hancock (1998) the embryonic duration of caridean shrimps is a curvilinear function of temperature: as the temperature increases, the rate of embryonic development increases up to a certain temperature. Most atyid shrimps hatch from the egg, superficially resembling very small adults (Day *et al.*, 2001).

In South Africa, rivers are considered important freshwater ecosystems, and many are recipients of both point and non-point source pollution (DWAF, 1997). *Daphnia pulex and Daphnia magna* have been widely used as standard test organisms for toxicity testing because they fulfil many of the requirements that are considered when selecting appropriate organisms. However, shortcomings considered are that *D. pulex* and *D. magna* are found predominantly in standing waters. There is therefore a need to produce data on how indigenous species respond to pollutants in their riverine environment.

*Caridina nilotica* has been identified as a potential standard toxicity test organism for producing ecologically relevant toxicity test data, as it is widely distributed and easy to find and it occurs in flowing waters (Hart, 1983). It is an indigenous species which can be easily cultured and maintained in the laboratory (Muller *et al.,* in press) and is also ecologically important (Hart, 1980). Toxicity tests have been developed for acute toxicity tests for neonate, juvenile and adult stages, and chronic test methods for (presumed) sensitive partial lifecycle test using young juveniles to early adults (Muller *et al*., in press), but there is an urgent need to develop a chronic toxicity test method using early life history stages.

Embryonic development as an ecotoxicity endpoint is an early life history stage test. Monitoring embryonic development as an ecotoxicity endpoint has been applied to various organisms (Rand, 1995). Fish embryo-larval assays and Frog Embryo Teratogenesis Assay, *Xenopus* (FETAX) are laboratory assays designed

specifically to investigate the toxicity of chemicals to the developing embryo and these are routinely employed in the toxicity characterization process (Kast-Hutcheson *et al.*, 2001). During the period of embryonic development cellular and molecular processes operate to generate a complex multicellular organism from a zygote. These processes are sensitive and easily perturbed by many chemicals (Rand, 1995). The use of embryonic development in routine and regulatory toxicity tests is currently being considered for *C. nilotica* (Muller *et al.,* in press).

## 1.5 Aims and objectives

The aims of this research were to:

- Determine embryonic development of *Caridina nilotica* under laboratory culturing conditions at the Institute for Water Research.
- Identify stages in embryonic development which could be used as quantifiable experimental endpoints in toxicity tests.
- Test the effects of three selected reference toxicants on these identified developmental stages in order to assess whether monitoring embryonic development in *Caridina nilotica* could be considered a suitable early life stage (chronic) toxicity test method.

# CHAPTER 2

## General materials and methods

A culture of freshwater shrimp was started in 1998, with *Caridina nilotica* collected from an unimpacted site, Mpisini stream (Richards Bay, KwaZulu-Natal). This chapter describes the methods for culturing *Caridina nilotica*, and obtaining embryos for experimental purpose. A description of the experimental chamber necessary to undertake the research is also provided. Experimental details for the toxicity tests can be found in chapter 4.

### 2.1 Laboratory culture maintenance

Shrimps of all ages were maintained in 30L (60x30x30cm) glass tanks according to a protocol developed and refined by UCEWQ-IWR Rhodes University, Grahamstown over the past seven years ( Muller *et al.*, in press). Each tank is equipped with an automatic aquarium heater, which is set at 24-26℃ and the temperatures in the tanks are recorded daily. Shrimp cultures receive natural light which is supplemented with (Biolux®) artificial light (12hr:12hr light:dark). During short day lengths (winter), the artificial light regime remains fixed to a 12 hour day length. The tanks are filled with dechlorinated tap water and aerators in each tank ensure that there is sufficient dissolved oxygen in the tanks. External biological filters act additionally to circulate the water through the tank. There is also a water circulating pump which is a biological filter and circulates the water in the tanks. It is recommended that the inlet of the filter is covered with a fine-meshed cloth; this prevents neonates from being drawn into the filter (Muller pers. com.). A net is suspended in the middle of the tank to act as a vertical surface for the shrimps to rest on.

Dissolved oxygen, pH and electrical conductivity are monitored and recorded weekly and if these water quality parameters are outside the set breeding conditions (5<pH<8, DO>5mg/L, 40mS/m<EC<60mS/m), dechlorinated or

deionised water (as appropriate) is used to restore the water quality. The body and the appendages of decapods are supported and protected in a firm or rigid exoskeleton of chitin, a carbohydrate polymer (Hart *et al.*, 2001). Often the chitin is impregnated with minerals, especially calcium. Concentrations of calcium dissolved in the medium in which the decapods live may influence their occurrence in freshwater (Hart *et al.*, 2001; Landau and Laufer, 1999); as calcium is required for exoskeleton formation, crushed shells are added in the biological filter. Higher levels of calcium should be avoided though because in high concentrations calcium appears to interfere with the bioavailability of phosphorus, which is also required for exoskeleton formation (Davis and Gatlin, 1991).

Gravid females were removed from the breeding tanks and placed in rearing tanks until the eggs hatch. When the eggs hatch, all the neonates and the adult females not used for experimental purposes were randomly returned to the breeding tanks. When the neonates reach the sexually mature stage they can breed with the older shrimps which are female, the young or small shrimps are males (Hart *et al.*, 2001, Schram, 1986). Individuals transform from male to female with increasing age (Okuthe *et al.*, 2004). Tanks were checked twice weekly for gravid females to ensure continued production of test organisms (neonates and older individuals) of known ages (Muller *et al*., in press).

Laboratory cultures of the *C. nilotica* were fed a general diet of a mixture of spirulina flakes and Tetramin® flake food (Muller pers. comm.). The food was placed in the water near the outlet of the filter so that the flake food is distributed by the flow. The feeding was monitored; if the guts were mostly full by the end of the day, no more food is given, but if the guts were mostly empty, more food is provided (Muller *et al*., in press). Faecal pellets which the animals scrape for diatoms are removed once a week as they can make the water become unclear (Muller pers. comm., 2005).

## 2.2 Experimental chambers

Using gravid females in toxicity test solutions places unnecessary burden on the cultures because breeding females cannot be returned to cultures after exposure to test solutions, and it takes three months to rear out breeding females (Muller pers. comm.; Slaughter, 2005). In addition, removing eggs daily from the females for examination could cause unnecessary stress to the female and lead to the females dropping the eggs. It was therefore necessary to establish whether there was a way to allow eggs to develop independently to parental female.

It was observed and reported that females of the *C. nilotica* carry their eggs in the brood pouch (see Fig. 2.1). The brood pouch is formed by pleopods which carry the eggs until hatching (Schram, 1986). The eggs are constantly palpated by the females using their pleopods, therefore an apparatus which could provide similar conditions to those in the brood pouch (i.e. gentle agitation, movement and oxygen) would allow development of embryos, while facilitating ease of removal of eggs for observations and measurements during experiments. This necessitated the development of an experimental chamber (Fig. 2.2).

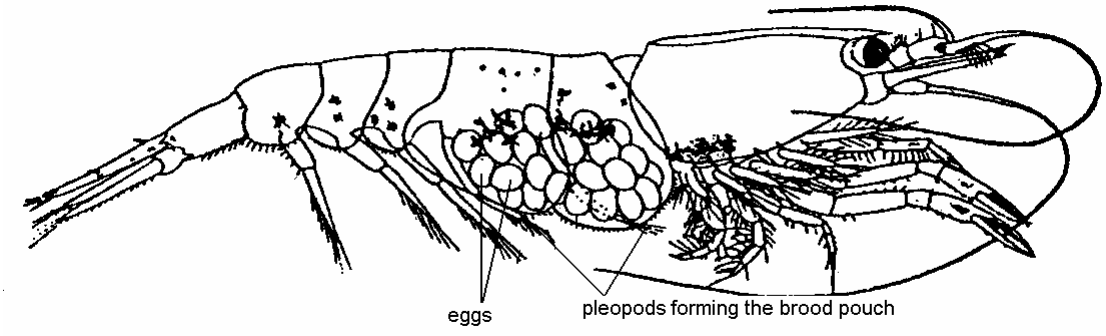


**Fig. 2.1** *Caridina nilotica* carrying the eggs in a brood pouch (Hart *et al*., 2001)

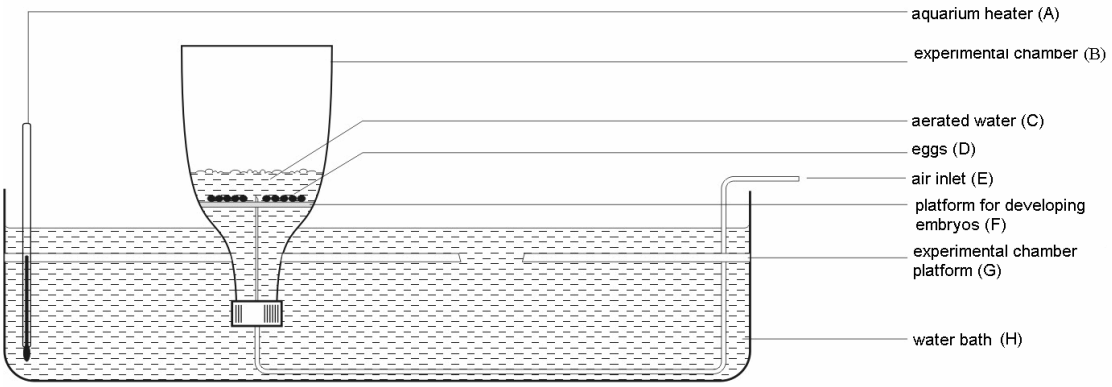


**Fig. 2.2** Experimental brood chamber for the development of the embryos which simulates the brood pouch of *C. nilotica*

Inverted glass flasks with their bottoms cut off formed the chamber (Fig. 2.2 (B)). A tube with a micropipette on its end was inserted through the bottom of the glass chambers and connected to an air pump through a set of valves which regulated the air flow (E), to provide constant and gentle aeration to the embryos. The brood chambers were sealed to ensure no fluid exchange between the water bath and the experimental brood chamber. This sealed unit held the eggs for the duration of the experimental period. The embryos were placed on a fine mesh gauze (F). Holes made in a Perspex sheet (G) held the experimental brood chambers.

A water bath (H) was filled with dechlorinated tap water, and it was maintained at 24ºC±0.5 using an aquarium heater (Fig. 2.2 (A)). This is the temperature the cultures of *C. nilotica* are maintained at, and air bubblers were connected to provide an even distribution of heat throughout the water bath. The glass chambers were placed in the water bath (using the platform (G)), to a depth that would ensure that solution temperature in the experimental chamber was kept constant by ensuring that there was enough water in the water bath surrounding the chambers. The heat from the water bath was transferred to the chambers, the temperature measured (Fig. 2.3) in the chambers was kept at a range of 23.5 -24.5ºC. Water quality parameters such as pH, Dissolved Oxygen (DO) and Electrical Conductivity (EC) were measured weekly inside the experimental chambers. Results of measurements taken from numerous chambers for the

duration of embryonic development showed that pH, DO and EC remained within the specifications for the breeding cultures (Figs. 2.4, 2.5 and 2.6) ( 5<pH<8, DO >5mg/L, 40mS/m<EC<60mS/m) (Muller *et al*., in press). The pH ranges were from 7.03 to 7.8, the DO ranged from 5.1mg/L to 7.2 mg/L and the EC ranged from 20.3mS/m to 49.4 mS/m.

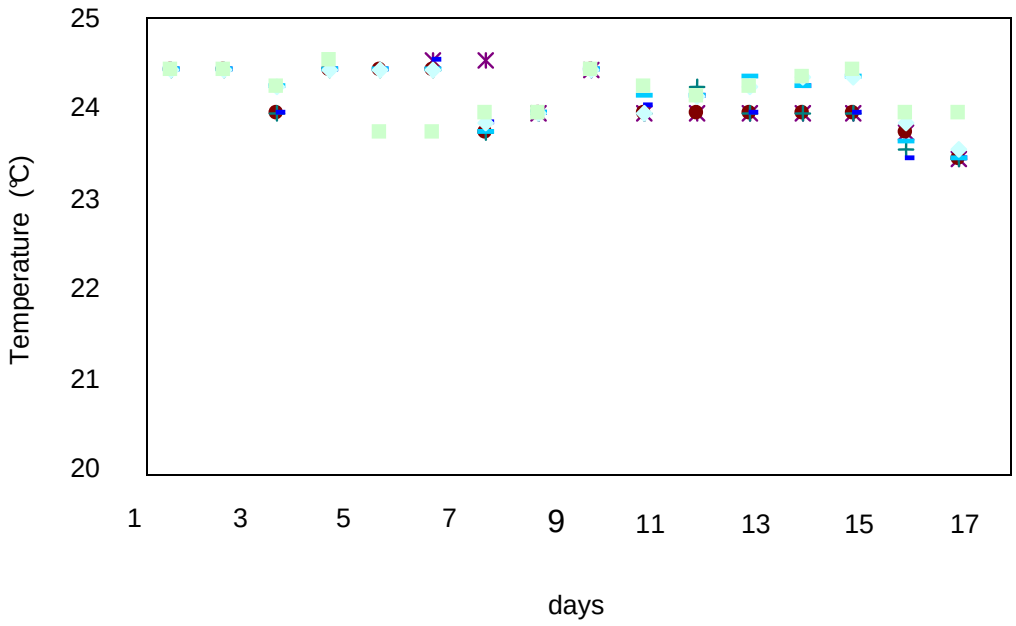


**Fig. 2.3** Daily temperature (℃) measurements in the brood c hambers for the duration of the development of the embryos of *C. nilotica*. The symbols represent different experimental brood chambers

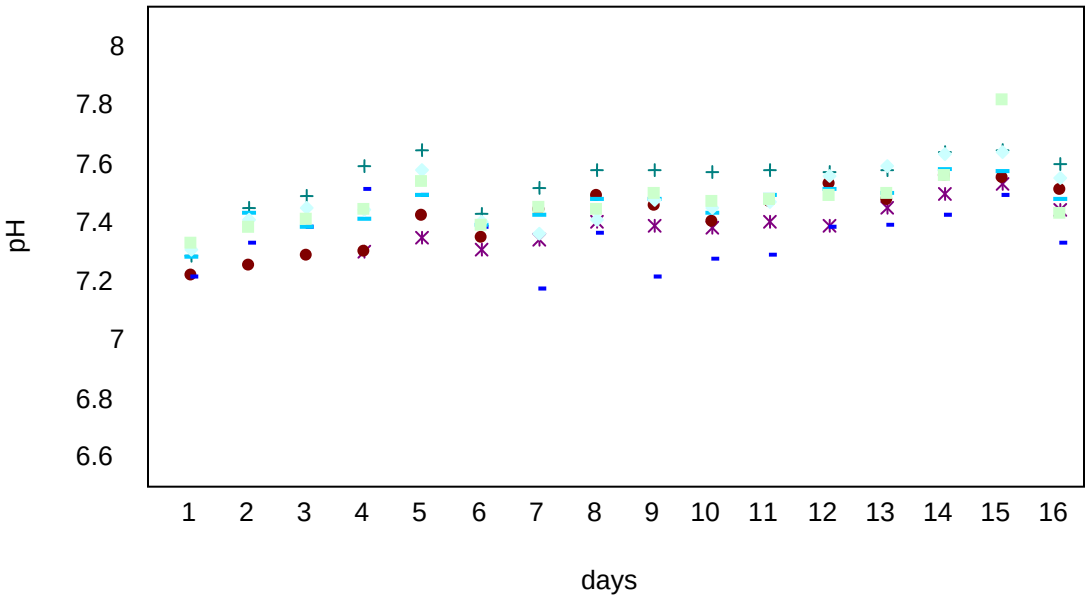


**Fig. 2.4** Daily pH measurements in the brood chambers for the duration of the development of the embryos of *C. nilotica*. The symbols represent different experimental brood chambers

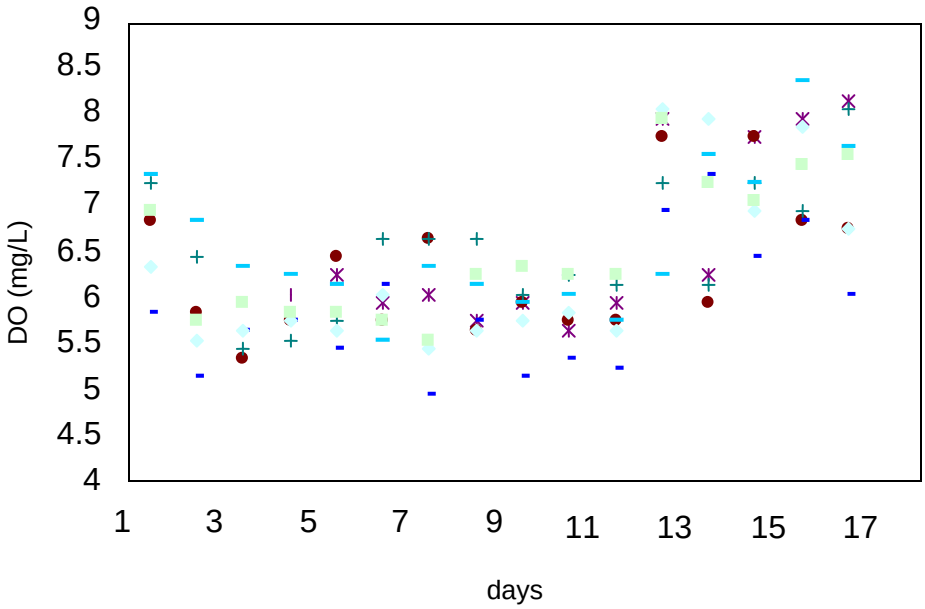


**Fig. 2.5** Daily dissolved oxygen (DO; mg/L) measurements in the brood chambers for the duration of the development of the embryos of *C. nilotica*. The symbols represent different experimental brood chambers

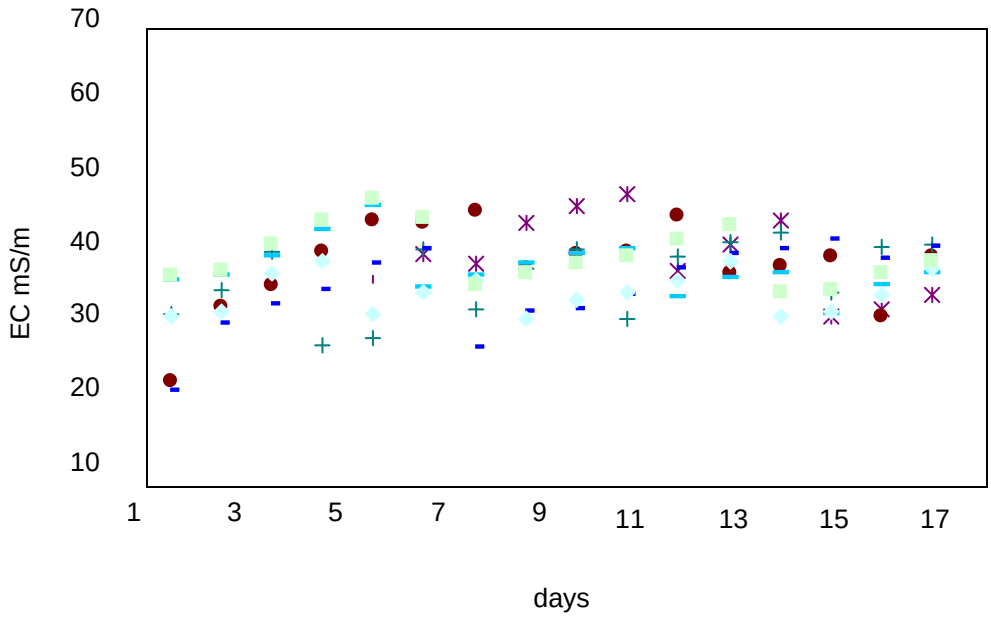


**Fig. 2.6** Daily electrical conductivity (EC; mS/m) measurements in the brood chambers for the duration of the development of the embryos of *C. nilotica*. The symbols represent different experimental brood chambers

## 2.3 Embryo collection

In order to obtain embryos of known age, eggs were removed from gravid females which had been gravid for less than 24 hours. The afternoon prior to commencement of a test, all gravid females were removed from each of the tanks, allowing collection of gravid females with eggs less than 24 hours on the following day. The newly spawned eggs were then extracted gently from the pleopods of the *C. nilotica* using a soft paintbrush, and eggs were randomly distributed to the experimental brood chamber by placing them gently on the mesh (Fig. 2.2).

## 2.4 Experimental procedure

Initially, daily development of the embryos was examined, and once the developmental stages were determined the observations could be undertaken at set intervals which are described in Table 2.1. At set intervals, eggs of known age were removed from the experimental chambers for examination. Eggs from each chamber were removed and placed in a Petri dish with water and live specimens were examined under the dissecting microscope (12x magnification). The live specimen made it easier to see the different features inside the embryo than when the specimen was fixed in 4% formalin. The developmental stages were recorded using an Olympus 4040 Zoom digital camera (Olympus American Inc., USA) attached to a light microscope. The electronic images were saved to a computer in order to record the measurements of identified developmental features by outlining the area of the features that needed measuring using a programme AnalySIS by Soft Imaging Systems (SIS). The length, width, length:width ratio and the area of the morphological features were measured. The measurements were saved to a spreadsheet (Excel) for further statistical analysis.

Embryonic development was measured by using a stereomicroscope, noting the shape of the egg, the quantity of yolk, the space occupied by the embryo, and the first appearance and subsequent growth of the eyes, appendages and other structures. Eggs that were dead were removed to prevent spreading of the fungi (Rand, 1995). They were easily identified as they were opaque and yellow in colour. The features of embryonic development identified in Table 2.1 were measured during toxicity experiments (details of how these stages were identified are explained in Chapter 3).

Table 2.1 The development features which were identified as potential experimental toxicity endpoints. The ages refer to the age of the embryo when the specified developmental features could be distinguished

| **Age (hrs)** | **Feature** |
|---|---|
| 24 | egg and yolk |
| 72 | antennae, antennules and optic rudiment |
| 144 | caudal papilla |
| 216 | eye and carapace |
| 288 | heartbeat and pleopods |
| 336 | development of all the somatic features |
| 408 | hatching |

## CHAPTER 3

## Embryonic development of *Caridina nilotica*

### 3.1 Introduction

As part of on-going research to investigate the use of indigenous riverine organisms as toxicity test organisms, a number of potential species were identified (Goetsch and Palmer, 1997; Haigh and Davies-Coleman, 1997). The freshwater shrimp *C. nilotica* is currently being evaluated as a potential indigenous test organism for use in regulatory aquatic toxicity tests alongside other standard, but non-indigenous, toxicity test organisms (Muller *et al.*, in press). The selection of a test organism is critical to the development of chronic toxicity test method. The test organism has a major influence on the relevance, success, and interpretation of a test (Rand, 1995).

*Caridina nilotica* was selected because it can be reared successfully under laboratory conditions at the UCEWQ (IWR, Rhodes University) and breeding is accomplished any time of the year (Hart, 1981). The culturing and breeding protocols of *C. nilotica* have also been developed (Muller *et al.*, in press).

However, few complete detailed studies of atyid embryonic development have been made (Chinnaya, 1974; Hancock, 1998; Nair, 1949, Nazari *et al.*, 2000) and very little is known about embryonic development of *C. nilotica*. Hart (1980) described the embryonic durations and post-embryonic growth rates of *C. nilotica* under laboratory and experimental field conditions. The embryonic durations and intermoult intervals decreased with increasing temperature (Hart, 1980).

The eggs of *C. nilotica* are oblong in shape and greenish-brown or greenish-yellow in colour throughout development, similar to those of *Caridina laevis* (Nair, 1949). The egg of *C. nilotica* resembles that of the large majority of the other crustaceans such as *Caridina laevis*, *Palaemonetes argentinus*, *Macrobrachium olfersi*, *Macrobrachium potiuna*, and *Palaemon pandaliformis*, in that they are typically centrolecithal (Nair, 1949; Nazari *et al.,* 2000; Müller, *et al.,* 2004), which means that the yolk food is at the centre of the egg.

Hancock (1998) divided egg development of *Paratya australiensis* (Atyidae) into 4 phases (without specifying the corresponding developmental ages):

- embryonic development not visible;
- embryo visible, cephalic lobes not extending halfway along side of eggs;
- embryo eyes visible;
- embryo with eyes fully formed and round and with only a small amount of yolk present.

The embryonic duration of this species was found to be related to temperature (Hancock, 1998). Other authors have tried to describe the daily developmental stages of other related species (Chinnaya, 1974, Lavarias *et al.*, 2002, Müller, *et al.,* 2004, Nair, 1949, Nazari *et al.*, 2000). Lavarias *et al.* (2002) even went as far as measuring the area, perimeter, maximum and minimum diameters of the yolk sac, egg coat and eye. The stages identified in these studies formed the basis of this research.

Therefore, the aims of the research presented in this chapter were to:

- determine embryonic development of *Caridina nilotica* under current laboratory culturing conditions and
- identify stages in embryonic development which could be used as quantifiable experimental endpoints in toxicity tests.

## 3.2 Materials and methods

### 3.2.1 Embryonic development

Before the actual embryonic development of *C. nilotica* was monitored, gravid females with newly spawned eggs were put in separate tanks to determine the duration of the embryonic development at 24℃±0.5 ( the prevailing culture conditions). It was determined that the development took 17 days, confirming earlier observations (Muller, pers. comm.). In order to determine whether the eggs were all in the same stage of development, the eggs were examined under the microscope before the start of the experiment. Experimental brood chambers filled with dechlorinated tap water were placed in a water bath with a temperature of 24℃±0.5. Newly spawned eggs were then extracted from gravid females using a soft paintbrush (Chapter 2). Eggs from the same clutch were placed in the same brood chamber. A total of 17 gravid females, with clutch sizes ranging from 19 to 60 eggs (Table 3.1) were used.

The daily embryonic development of *C. nilotica* was monitored and measured at 24℃±0.5. The brood chambers were marked and daily all the eggs from one chamber were removed to provide a 24 hourly record of embryonic development. The developmental ages were monitored under the dissecting microscope. The developmental ages were recorded using an Olympus 4040 Zoom digital camera (Olympus American Inc., USA) attached to a light microscope. The electronic images were saved onto a computer to record the measurements of the features by outlining the area of the features that were to be measured using a

programme AnalySIS by Soft Imaging Systems (SIS). The length, width, length:width ratio and the area of the morphological features were measured. These data were then used to identify developmental stages which could be used in the chronic toxicity tests.

### 3.2.2 Variability in embryonic development between embryos

Using the defined developmental stage at specific ages defined in Table 2.1, the differences or similarities between eggs from the same female (intrafemale) and then different females (interfemale) were evaluated.

#### Intrafemale variability in embryonic development

The variability in size of morphological features between eggs of the same clutch from a gravid shrimp was determined, following the same procedure reported for embryonic development. A single female's eggs were placed in each brood chamber filled with dechlorinated tap water at 24℃ ±0.5. On a given day all the eggs in a single chamber were removed and examined. The length, width, length:width ratio and the area of the egg, yolk, antennae, antennule, optic rudiment, caudal papilla, eye, carapace and pleopods were measured. The developmental stages were monitored and measured under the dissecting microscope. The developmental stages were recorded using an Olympus 4040 Zoom digital camera (Olympus American Inc., USA) attached to a light microscope. The electronic images were saved to a computer to record the measurements of the features by outlining the area of the features that were to be measured, using a programme AnalySIS by Soft Imaging Systems (SIS).

#### Interfemale variability in embryonic development

The variability in size of features between eggs of different clutches from 10 different females was determined. Ten experimental brood chambers were placed in the water bath at 24℃±0.5 and eggs from the same clutch were placed

into the same chamber; a minimum of 30 eggs was placed in each chamber. However, at a predetermined developmental age, 10 eggs were removed from each chamber, and developmental state at specific ages identified and measured. Developmental progress at three randomly selected ages was used to test inter individual variability. These were:

72hrs: development of the antennae, antennule and optic rudiment
216hrs: development of the eye
384hrs: development of all the features.

**Random variability**

Eggs of the same age but from different females were mixed together in the same brood chamber to assess whether mixing eggs from different females would result in greater or less variability than when all measures for a given development state are from a single female. The eggs were placed into three chambers. Daily, 10 eggs were removed from each chamber. The same procedure used to measure intrafemale variability was followed to measure random variability.

### 3.2.3 Statistical analysis

A one-way Analysis of Variance (ANOVA) test was used to compare the sizes of the features between eggs from the same female, between eggs of different females and between randomly mixed eggs. Intrafemale and interfemale variability in the feature measured were analysed using ANOVA in Statistica Version 6 (StatSoft, 2002). An ANOVA statistical method assumes that the data have less variability and that the data follow a normal distribution (Crane *et al.*, 2000). The reality of the assumption was explored using Kolmogorov-Smirnov & Lillifors test (StatSoft, 2002). Whisker plots were drawn to show variability between the eggs.

## 3.3 Results

### 3.3.1 Embryonic development

The number of eggs produced in a clutch ranged from 19 to 60 eggs (Table 3.1). The females were of unknown ages and the size of females was not measured.

Table 3.1 The number of eggs in each clutch from the gravid females of *C. nilotica* which were placed in the experimental brood chambers

| **Female** | **No of eggs** |
|---|---|
| Female 1 | 21 |
| Female 2 | 29 |
| Female 3 | 25 |
| Female 4 | 30 |
| Female 5 | 47 |
| Female 6 | 59 |
| Female 7 | 31 |
| Female 8 | 24 |
| Female 9 | 23 |
| Female 10 | 29 |
| Female 11 | 49 |
| Female 12 | 60 |
| Female 13 | 32 |
| Female 14 | 19 |
| Female 15 | 25 |
| Female 16 | 27 |
| Female 17 | 57 |

This variation may be related to the size of the females with smaller sized females producing fewer eggs than the larger females (personal observation). The complete embryonic development of *Caridina nilotica* was described based on external morphological changes and 7 development ages could be clearly identified, similar to those of the other studies (Chinnaya, 1974, Hancock, 1998,

Lavarias *et al.*, 2002, Nair, 1949, Nazari, 2000, Müller, *et al.,* 2004). Even though the developmental stages were monitored and examined daily, the results presented in this study are those ages where changes were first distinguished. The hours that are not presented only showed an increase in sizes of the features, and no new developmental features were observed. Clearly definable ages were identified, and these are listed in Tables 3.2 and 3.3 and shown in Figs. 3.1- 3.6. The length, width, length:width ratio and the area of the developmental features which were described in the developmental stages were measured (Tables 3.2 and 3.3). The eggs chosen to represent the stages in Figures 3.1-3.6 were those that most clearly represented the features of that particular developmental stage.

Table 3.2 The embryonic developmental ages of *C. nilotica* starting from spawning until egg hatching

| **Age of embryo (hrs)** | **Developmental ages** |
|---|---|
| 24 | At spawning the yolk mass is regularly distributed throughout the egg, with the chorion covering and protecting the egg. The yolk shrinks slightly before cleavage sets in during the first 24 hours and then it undergoes cleavage into different blastomere stages (see Fig.3.1). |
| 72 | The antennae, antennules and optic rudiment start to develop (see Fig.3.2). |
| 144 | The antennae and antennules have become long and pointed and well developed. The caudal papilla is developed (see Fig.3.3) and gives slight jerks. |
| 216 | All the appendages show slight movements. The whole embryo starts moving its body at intervals. The carapace of the embryo starts to develop. The pigmentation of the eye is visible (see Fig.3.4). |
| 288 | The pulsations of the heart are seen clearly. Small setae are developed at the tips of the thoracic appendages. Pleopods are visible (see Fig.3.5). |
| 336 | All the external characteristics of the embryo are visible, the development is complete and the embryo becomes larger. The yolk of the embryo is absorbed completely (see Fig.3.6). Heartbeat and movements of the embryo are faster than compared to 288 hours. |
| 408 | The larvae are released into the water by this day. The newly hatched animals have all the somatic features of the adult shrimp but are significantly smaller. |

Table 3.3 Measurements of the lengths (L) and widths (W) (mm), length:width ratios (L:W) and area (A) ($mm^2$) of the features of *C. nilotica* embryos at identified developmental stages. The values represent the means for each measured stage, with a sample size of 10 eggs from different females per measurement

| Age | egg | yolk | antennae | antennule | optic rudiment | caudal papilla | eye | carapace | pleopods |
|---|---|---|---|---|---|---|---|---|---|
| **24 hours** | | | | | | | | | |
| L | 1.2 | 1.2 | | | | | | | |
| W | 0.66 | 0.66 | | | | | | | |
| L/W | 1.81 | 1.81 | | | | | | | |
| A | 0.63 | 0.63 | | | | | | | |
| **72 hours** | | | | | | | | | |
| L | 1.2 | 1.15 | 0.19 | 0.14 | 0.2 | | | | |
| W | 0.62 | 0.62 | | | | | | | |
| L/W | 1.94 | 1.85 | | | | | | | |
| A | 0.64 | 0.62 | | | 0.0011 | | | | |
| **144 hours** | | | | | | | | | |
| L | 1.23 | 1.01 | 0.22 | 0.19 | 0.21 | 0.18 | | | |
| W | 0.67 | 0.64 | | | | | | | |
| L/W | 1.84 | 1.58 | | | | | | | |
| A | 0.69 | 0.6 | | | 0.0024 | | | | |
| **216 hours** | | | | | | | | | |
| L | 1.27 | 0.64 | 0.31 | 0.23 | 0.24 | 0.28 | 0.1 | 0.24 | |
| W | 0.7 | 0.66 | | | | | 0.03 | | |
| L/W | 1.81 | 0.97 | | | | | 3.33 | | |
| A | 0.75 | 0.4 | | | 0.009 | | 0.001 | | |
| **288 hours** | | | | | | | | | |
| L | 1.32 | 0.55 | 0.51 | 0.42 | 0.29 | 0.67 | 0.12 | 0.26 | 0.07 |
| W | 0.76 | 0.65 | | | | | 0.07 | | |
| L/W | 1.74 | 0.85 | | | | | 1.71 | | |
| A | 0.8 | 0.38 | | | 0.021 | | 0.005 | | |
| **336 hours** | | | | | | | | | |
| L | 1.36 | 0.21 | 0.64 | 0.49 | 0.32 | 0.85 | 0.13 | 0.28 | 0.09 |
| W | 0.79 | 0.64 | | | | | 0.11 | | |
| L/W | 1.72 | 0.33 | | | | | 1.18 | | |
| A | 0.86 | 0.18 | | | 0.04 | | 0.007 | | |
| **384 hours** | | | | | | | | | |
| L | 2.3 | 0 | 0.69 | 0.59 | 0.34 | 1.12 | 0.14 | 0.34 | 0.1 |
| W | 0.84 | 0 | | | | | 0.13 | | |
| L/W | 2.74 | 0 | | | | | 1.08 | | |
| A | 0.91 | 0 | | | 0.043 | | 0.008 | | |
| **408 hours** | | | | | | | | | |
| L | | | 0.71 | 0.61 | 0.35 | 1.17 | 0.15 | 0.36 | 0.11 |
| W | | | | | | | 0.14 | | |
| L/W | | | | | | | 1.07 | | |
| A | | | | | 0.044 | | 0.009 | | |

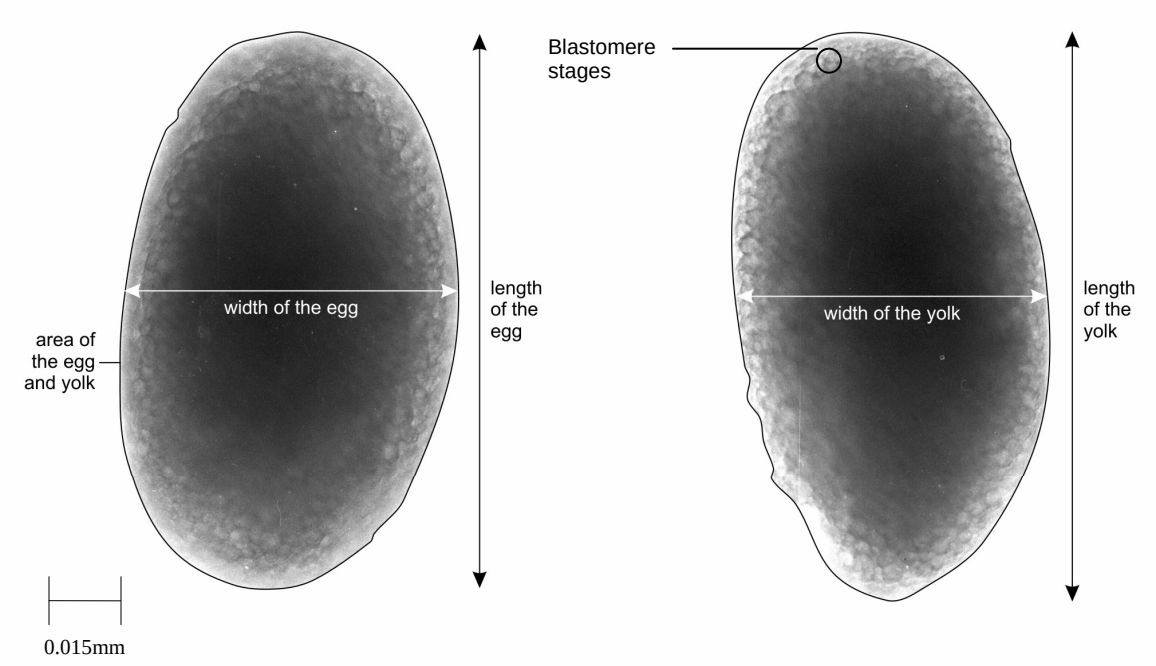


**Fig. 3.1** Embryonic development of *C. nilotica* at 24 hours with the lines indicating the length, width and area of the egg and the yolk shown by enclosed lines (Scale bar=0.015mm)

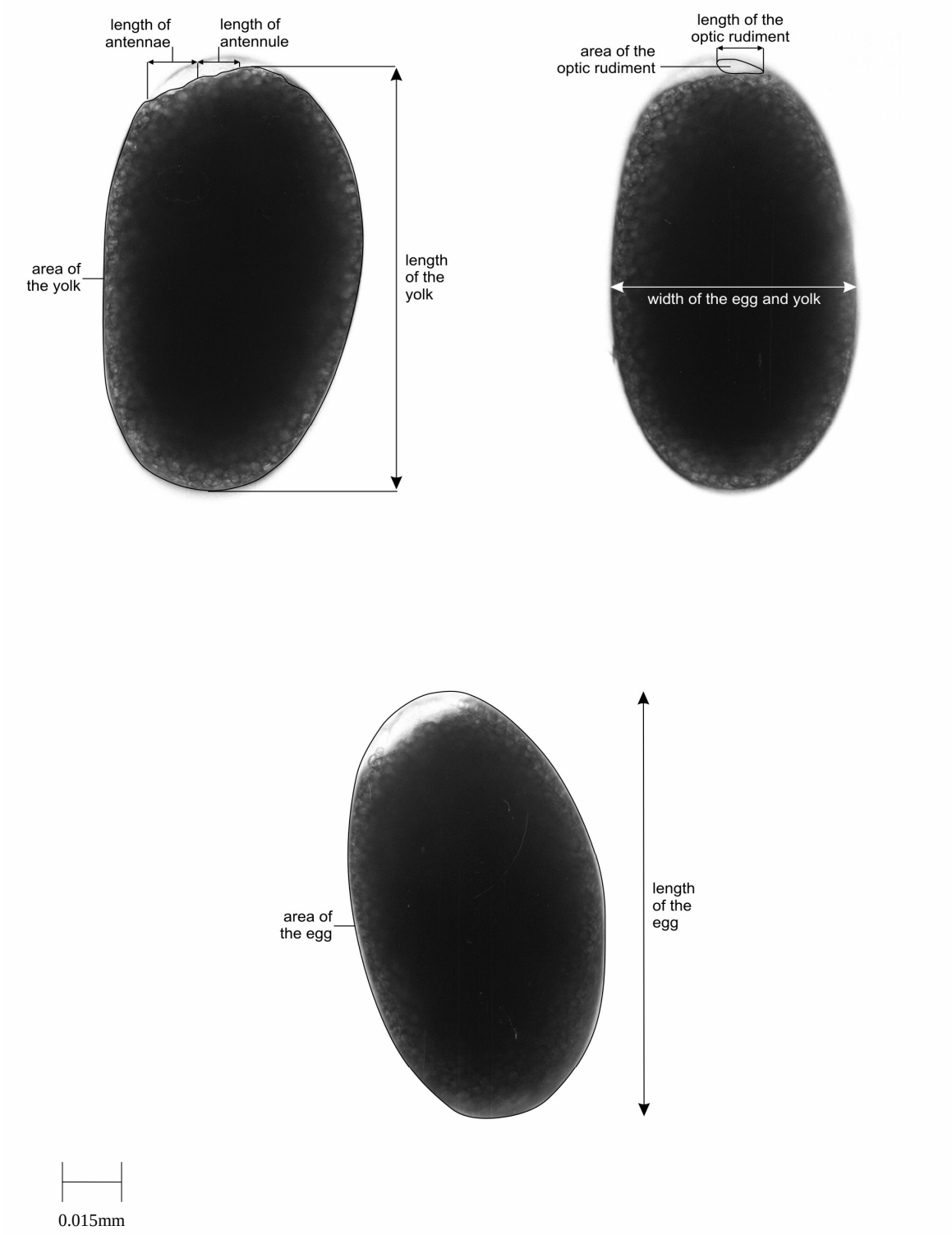


**Fig. 3.2** Embryonic development of *C. nilotica* at 72 hours with the lines indicating the length and width of the egg and yolk; length of the antennae, antennule and optic rudiment. Areas of the egg, yolk and optic rudiment are shown by enclosed lines (Scale bar=0.015mm)

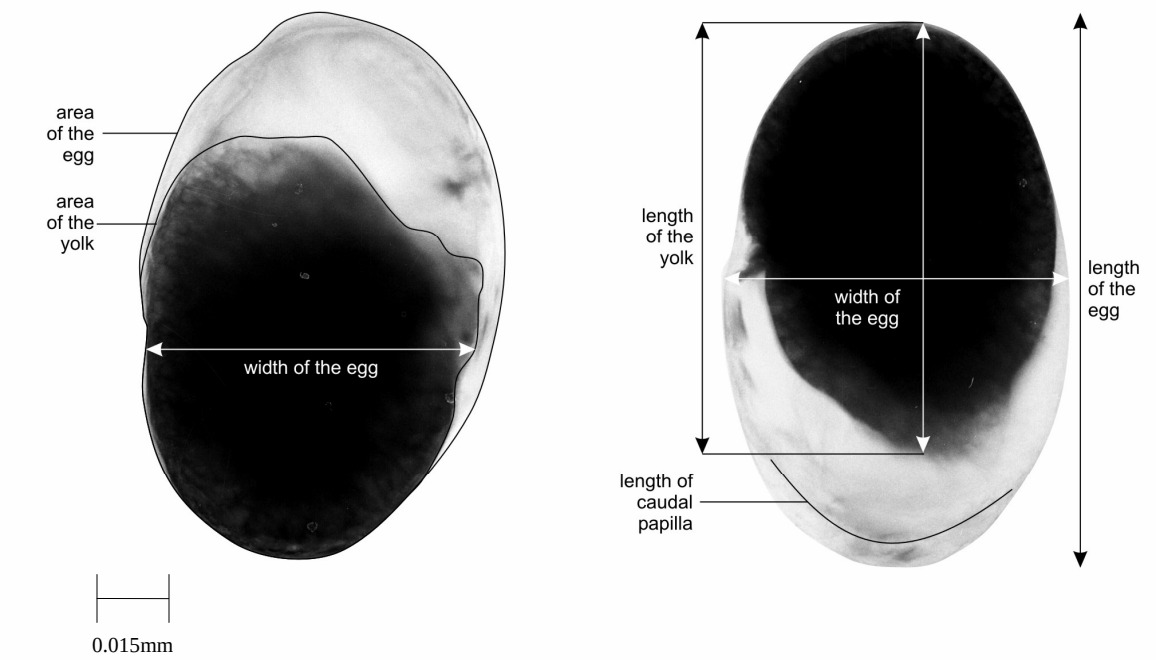


**Fig. 3.3** Embryonic development of *C. nilotica* at 144 hours with the lines indicating the length, width and area of the egg and yolk shown by enclosed lines, length of the caudal papilla (Scale bar=0.015mm)

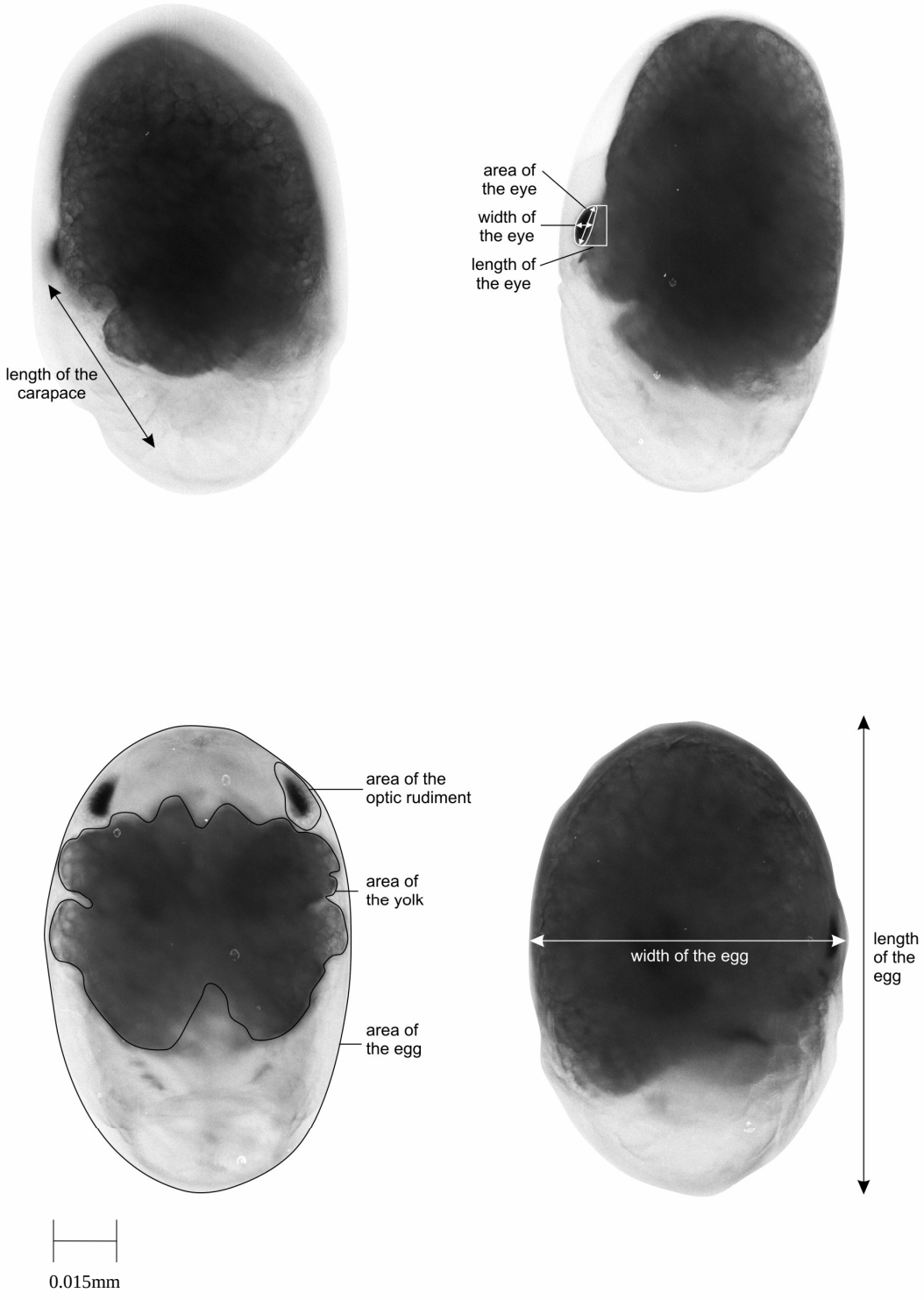


**Fig. 3.4** Embryonic development of *C. nilotica* at 216 hours with lines indicating the area, width and length of the eye; length and width of the egg; length of the carapace. Areas of the optic rudiment, yolk and egg are shown by enclosed lines (Scale bar=0.015mm)

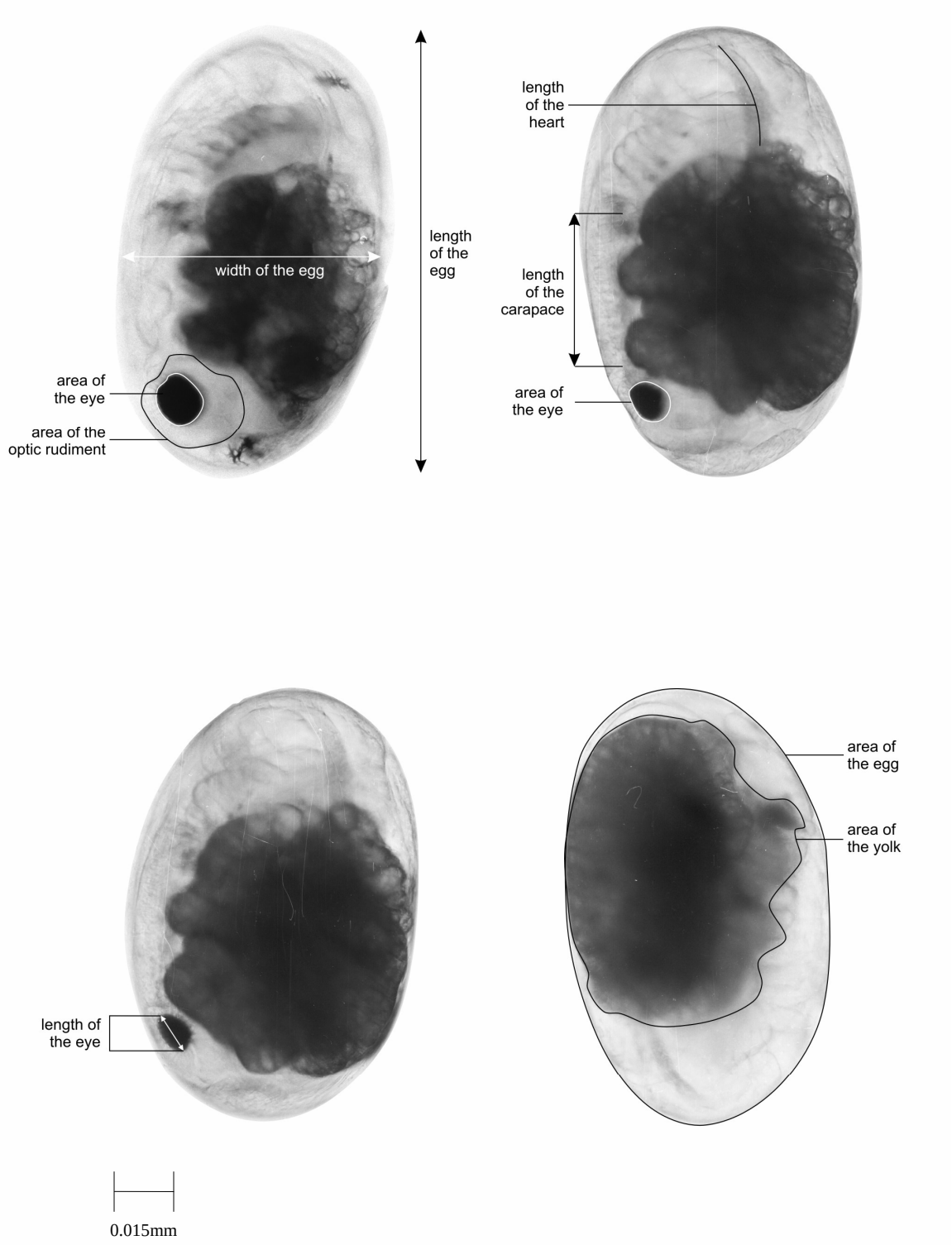


**Fig. 3.5** Embryonic development of *C. nilotica* at 288 hours with lines indicating width and length of the egg; area of the eye, optic rudiment, egg and yolk are shown by the enclosed lines; length of the heart, carapace and eye (Scale bar=0.015mm)

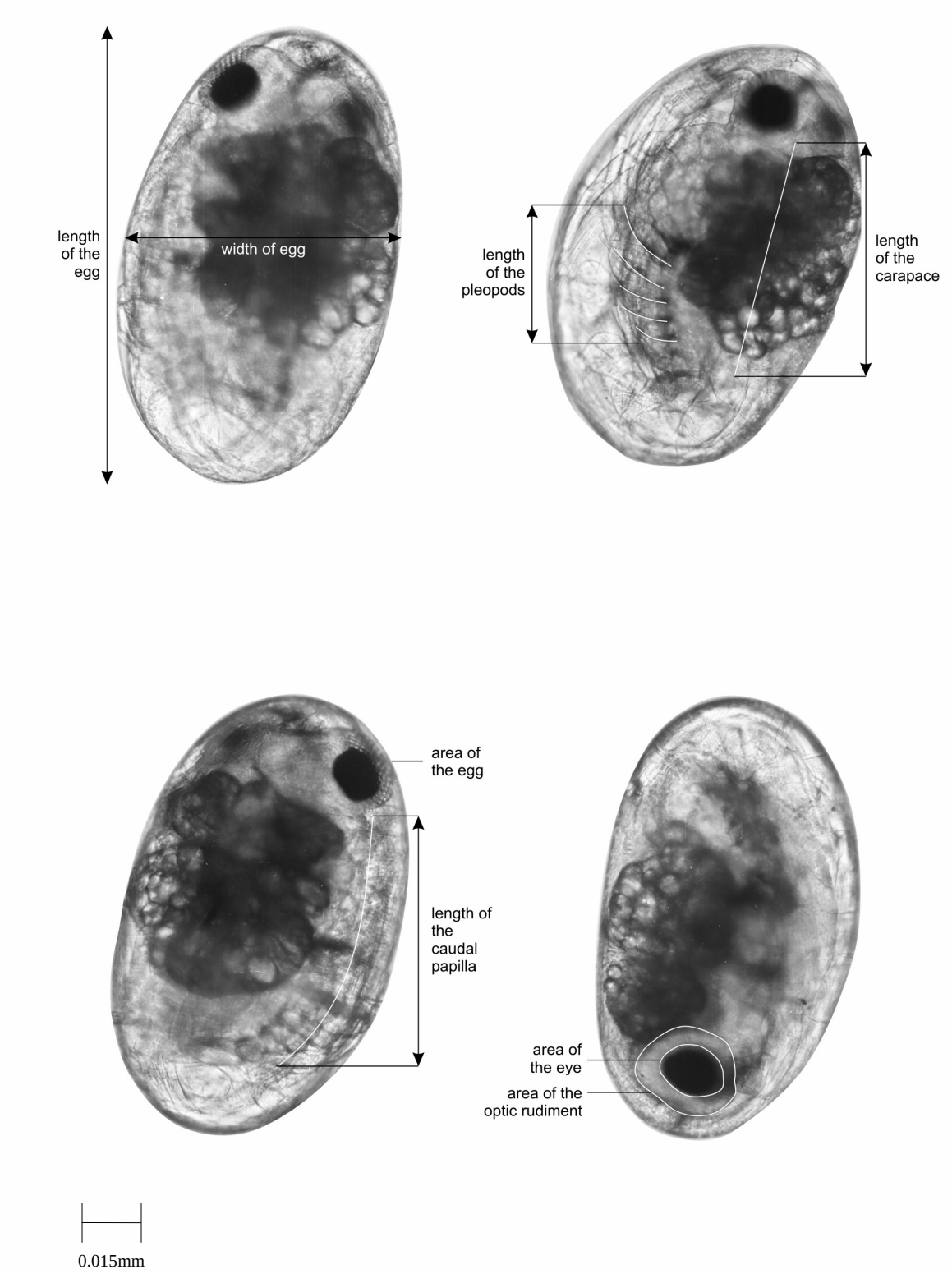


**Fig. 3.6** Embryonic development of *C. nilotica* at 336 hours with lines indicating length of the egg, pleopods, carapace and caudal papilla; areas of the egg, eye and optic rudiment shown by enclosed lines width of the egg (Scale bar=0.015mm)

The development of features was noted at 72 hours where the antenna, antennule and the optic rudiment are identified (Fig. 3.2). The embryo continues to grow after that with the antennae and the antennules starting to elongate. At 144 hours the caudal papilla starts to develop and was seen to exhibit small jerks, while the other features have increased in size (Fig. 3.3). The eyes appear at 216 hours of development (Fig. 3.4), but they become more visible and conspicuous at 240 hours and become more spherical before the juvenile hatches. At 288 hours the pulsations of the heart and the pleopods can be seen clearly (Fig. 3.5). The presence of caudal papilla and the heart is recognised by the movement they make from time to time. At 336 hours the embryo is more developed (Fig. 3.6) the yolk mass has diminished in quantity; the embryos hatch at 408 hours with the features similar to the adult *C. nilotica,* only smaller.

Yolk sizes decreased during development of *C. nilotica,* confirming the utilization of yolk as energy source (García-Guerrero *et al*., 2003). During the first 24 hours the blastomeres round off giving the embryo a characteristic grape-bunch appearance as can also be observed in *Caridina laevis* (Nair, 1949) which shows that cleavage is about to set in. At 48 hours there are no substantial changes that can be observed in the egg: the egg has increased in size and the yolk mass decreased.

Fig. 3.7 shows that the length of the egg increased over the duration of the developmental period. At 48 hours there was a larger mean egg length than at 72 hours although this difference was not significant. Fig 3.8 shows that the length of the yolk decreased over time and at 384 hours the yolk which the embryo feeds on is completely depleted. Figs. 3.9 to 3.10 show that the length of optic rudiment and the antennae increases over time. However, at 96 hours there was a slight decrease in the optic rudiment mean length to 0.19mm after which the optic rudiment continued to increase. There was a gradual increase in the mean length of the antennae until 192 hours, but after that the mean length increased sharply. Figs. 3.11 to 3.15 show that the length of the antennule, caudal papilla, eye, carapace and pleopods increased over time.

Although the pattern of increasing length of features and decreasing length of yolk was the same for the width, length:width ratio and the area, only the results of the measured lengths of the identified features are presented. For graphs depicting the widths, length:width and area of the measured features, refer to Appendix A.

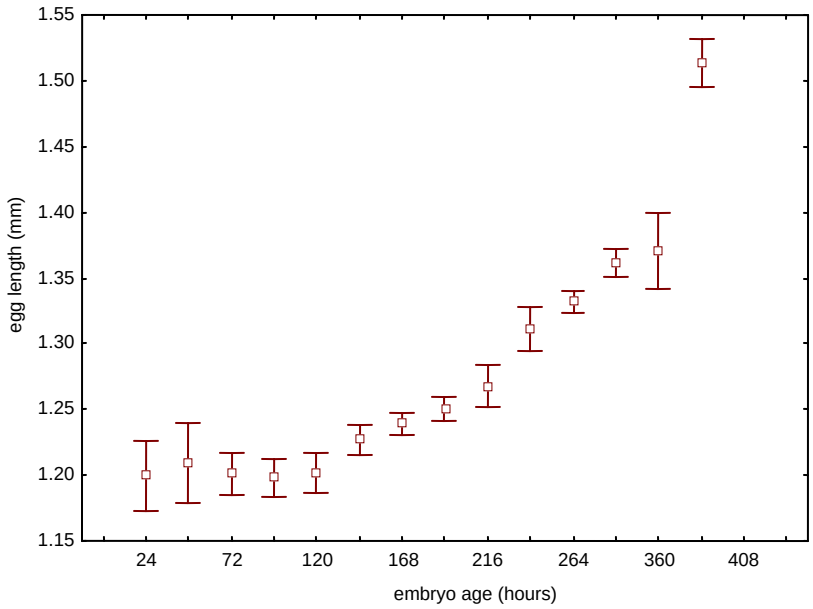


**Fig. 3.7** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the egg length (mm) of *C. nilotica* embryos over time

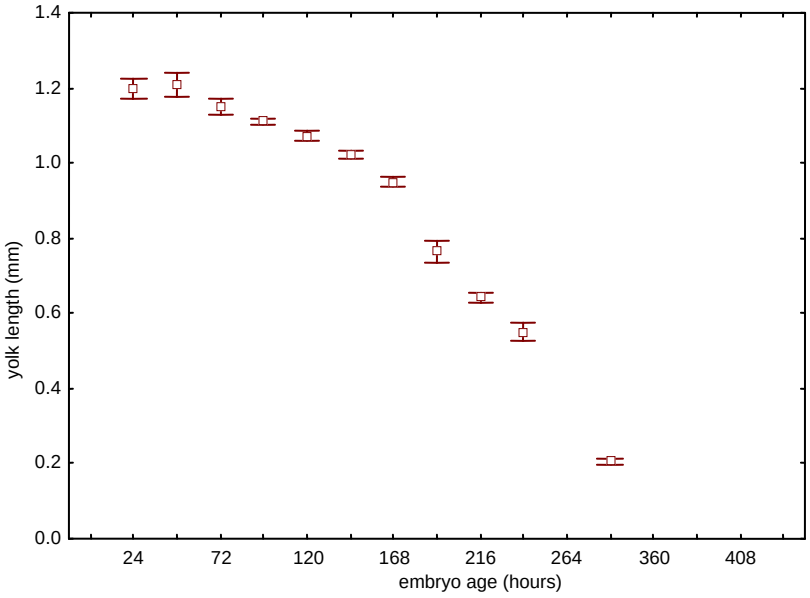


**Fig. 3.8** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the yolk length (mm) of *C. nilotica* embryos over time

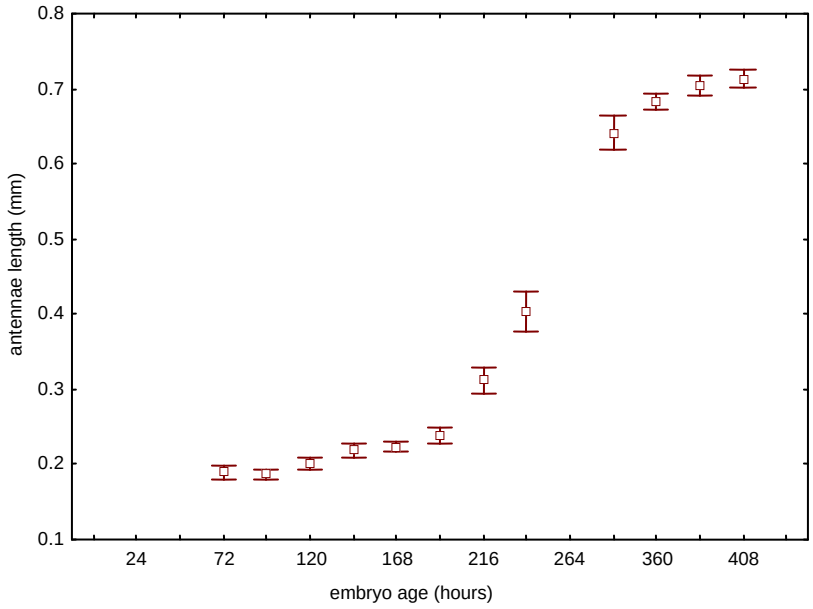


**Fig. 3.9** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the antennae length (mm) of *C. nilotica* embryos over time

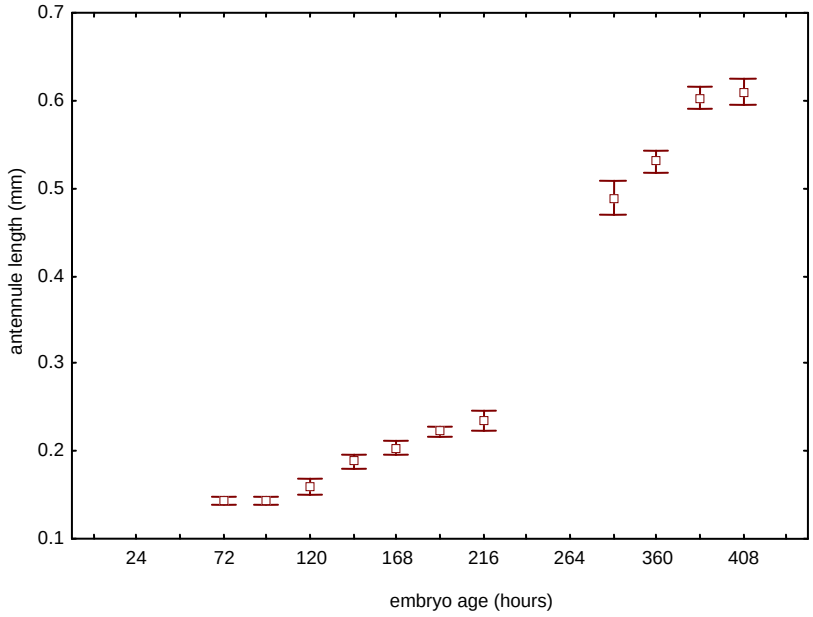


**Fig. 3.10** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the antennule length (mm) of *C. nilotica* embryos over time

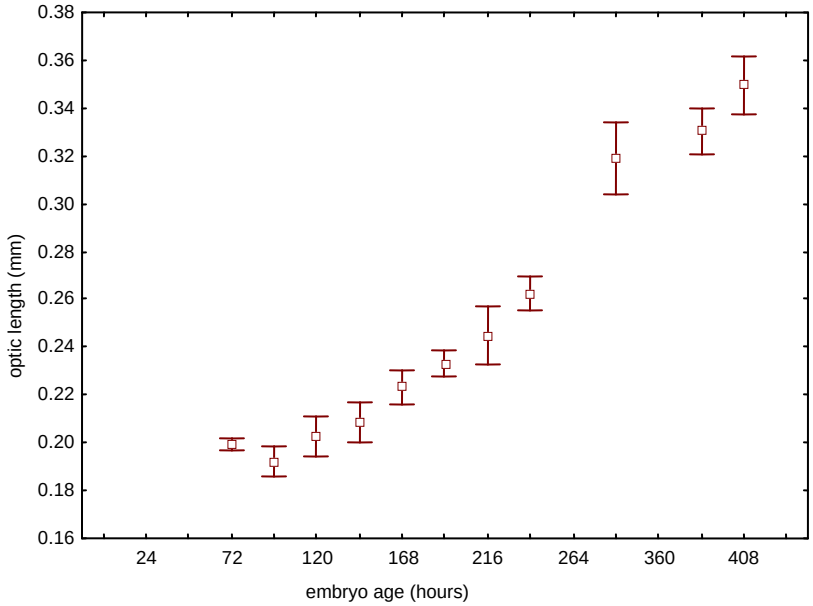


**Fig. 3.11** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the optic rudiment length (mm) of *C. nilotica* embryos over time

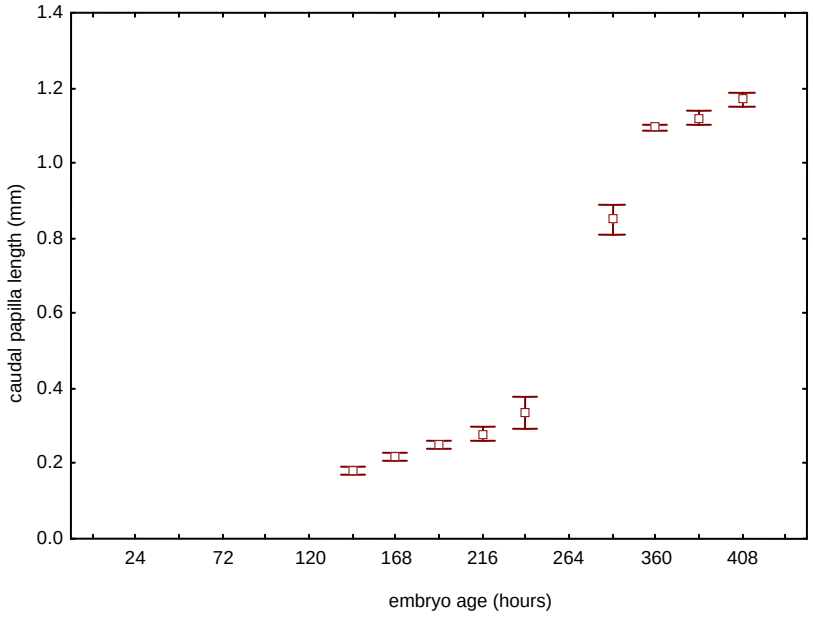


**Fig. 3.12** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the caudal papilla length (mm) of *C. nilotica* embryos over time

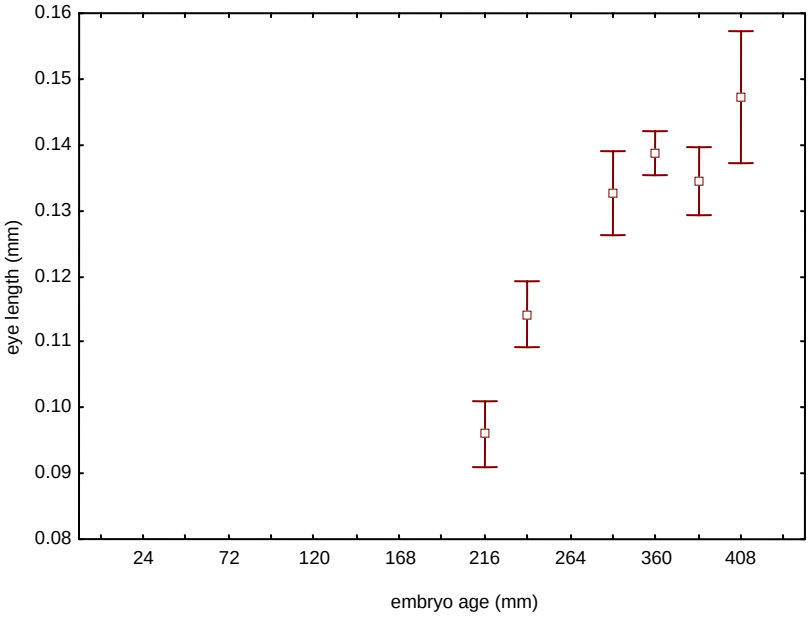


**Fig. 3.13** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the eye length (mm) of *C. nilotica* embryos over time

Based on the examination of embryonic development and the literature (Chinnaya, 1974, Lavarias *et al.*, 2002, Nair, 1949, Nazari, 2000, Nazari *et al.*, 2004), the following embryonic features and the associated ages of embryo were identified as potential experimental toxicity endpoints (Table 3.4)

Table 3.4 The ages which were identified as potential experimental toxicity endpoints. The ages refer to the age of the embryo when the specified developmental features could be distinguished

| **Age (hrs)** | **Developmental Feature** |
|---|---|
| 24 | egg and yolk |
| 72 | antennae, antennules and optic rudiment |
| 144 | caudal papilla |
| 216 | eye and carapace |
| 288 | heartbeat and pleopods |
| 336 | development of all features |
| 408 | hatching |

### 3.3.2 Variability in embryonic development

#### Intrafemale variability

Figures 3.16 to 3.22 show the intrafemale variability of embryonic development between the eggs (only linear values are presented). The results presented are those of the embryonic features which were identified as potential experimental toxicity endpoints (Table 3.4). The developmental features show an increase in size of length, except in yolk where there is a decrease in length. There were no significant differences between eggs from the same female, $p>0.05$. The mean lengths of the developmental features were similar to the measurements in Table 3.3 of the identified developmental ages.

Although only results for length measurements are presented, mean width, length:width ratios and area showed the same trend and are presented in Appendix A (Figs. A-C).

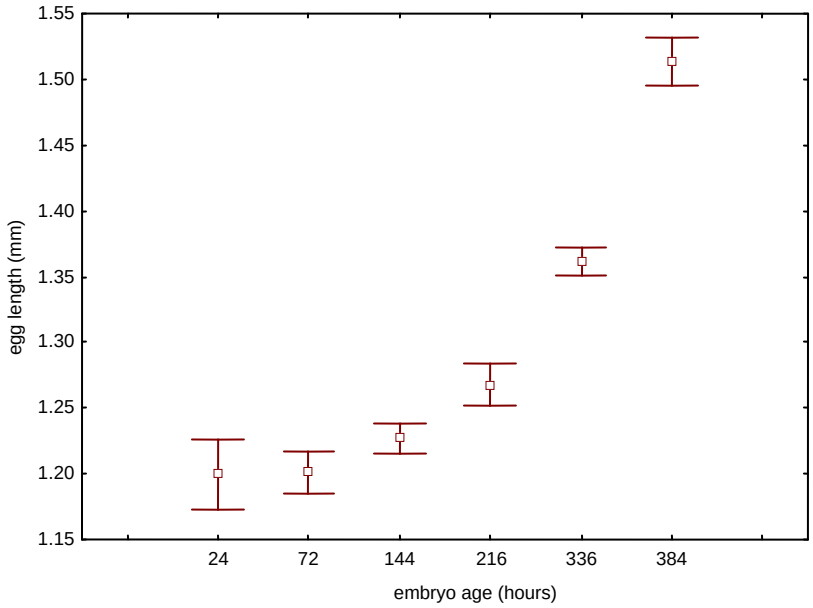


**Fig. 3.16** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the egg length (mm) of *C. nilotica* embryos over time

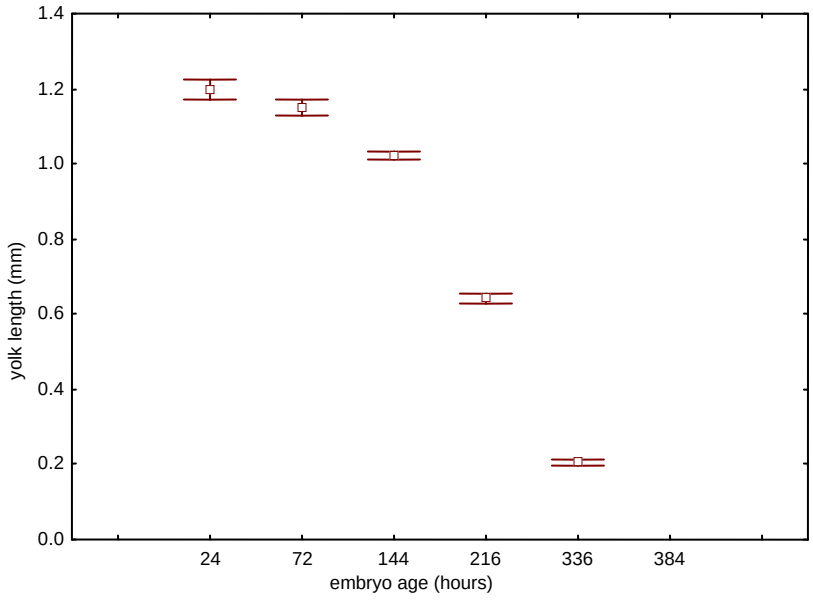


**Fig. 3.17** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the yolk length (mm) of *C. nilotica* embryos over time

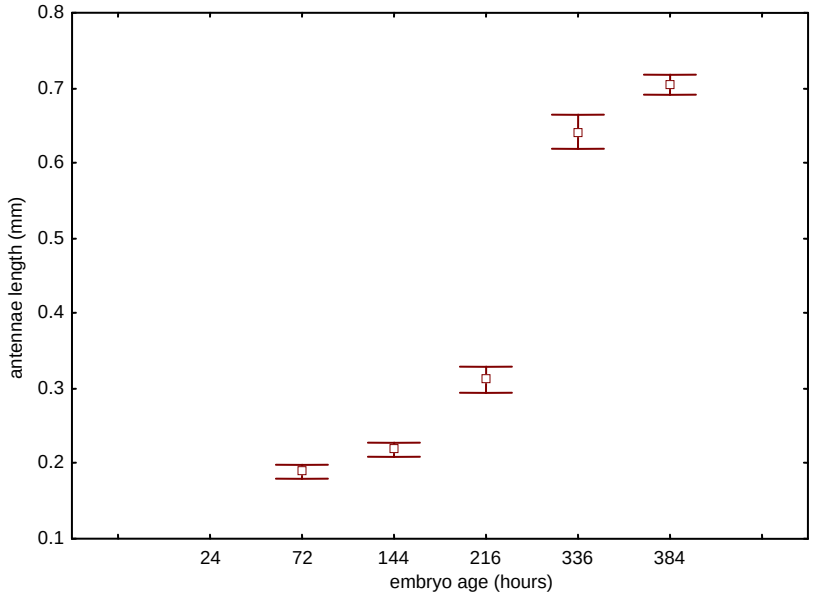


**Fig. 3.18** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the antennae length (mm) of *C. nilotica* embryos over time

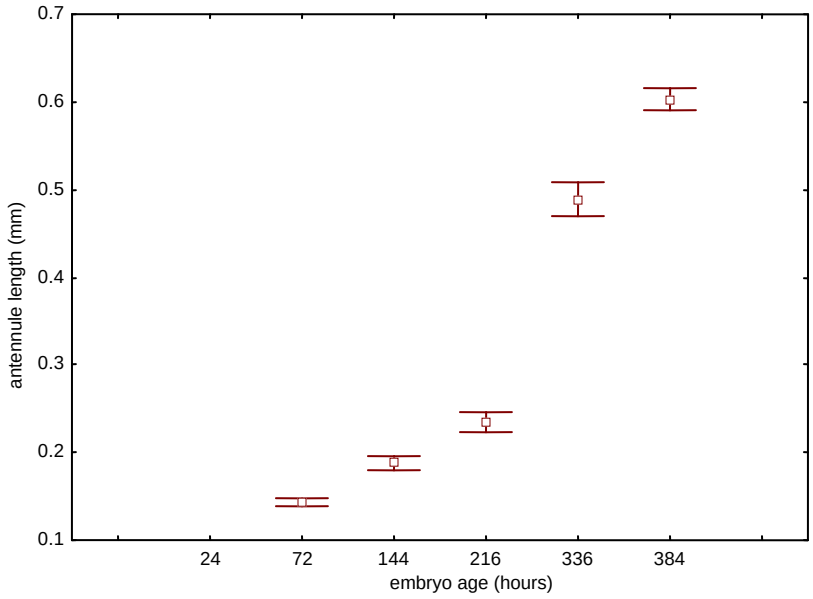


**Fig. 3.19** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the antennule length (mm) of *C. nilotica* embryos over time

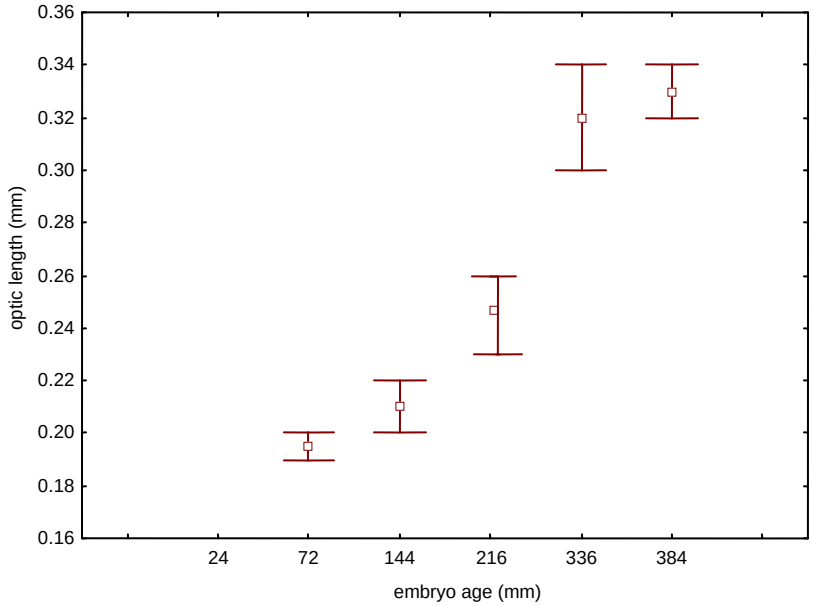


**Fig. 3.20** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the optic rudiment length (mm) of *C. nilotica* embryos over time

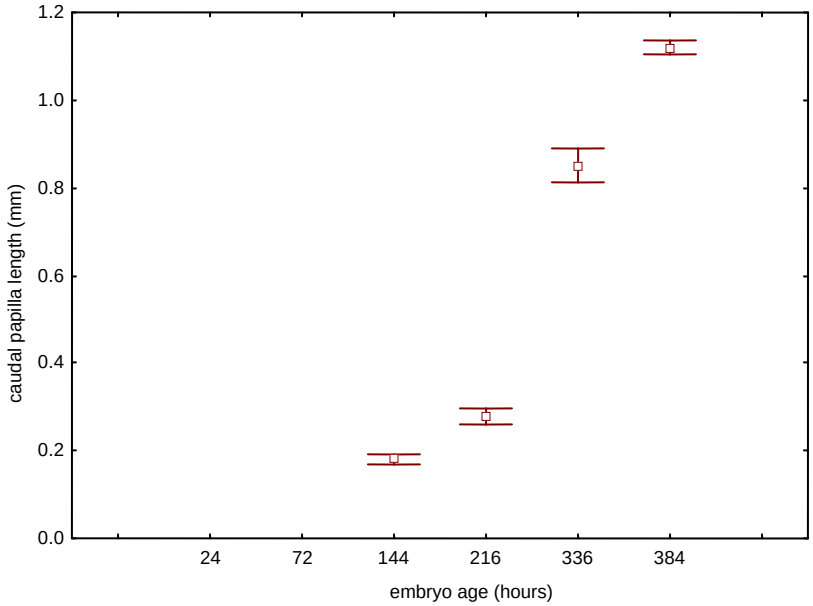


**Fig. 3.21** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the caudal papilla length (mm) of *C. nilotica* embryos over time

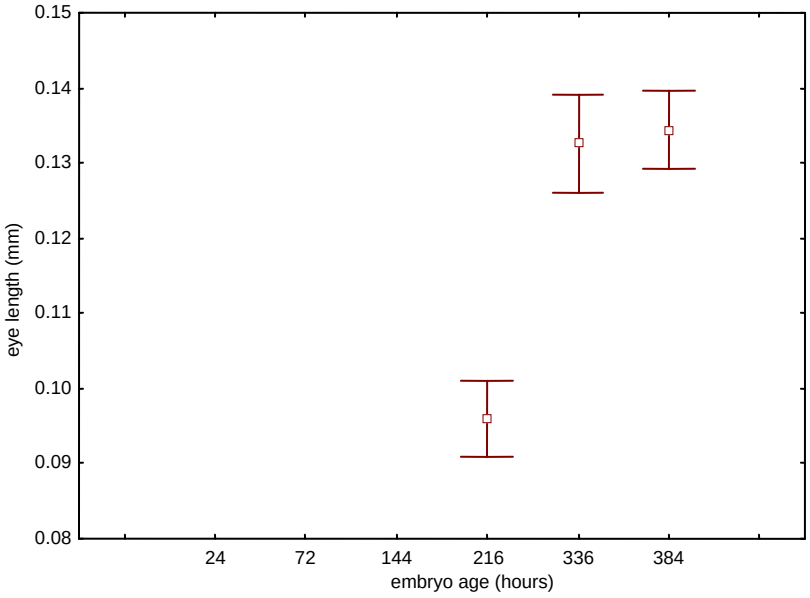


**Fig. 3.22** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the eye length (mm) of *C. nilotica* embryos over time

**Interfemale variability**

Figures 3.23 to 3.40 show the interfemale variability of embryonic development between different females for length measurements. The p-values were all greater than 0.05, indicating there were no significant differences between the stages of development of eggs of different females at any particular interval (Table 3.5). The results presented in Table 3.5 show the mean lengths of the measured features of embryos at the randomly selected intervals (72, 216 and 384 hours) for all 10 females. The mean lengths and the p-values of the different measured features are shown in Table 3.5. The low % coefficient of variation (CV%) indicates that there is an insignificant difference between the measured developmental features.

Although only results for length measurements are presented, mean width, length:width ratio and the area measurements followed the same trend as the mean lengths of the measured features and are shown in Appendix A (Figs. D-X).

Table 3.5 Mean lengths, p-values and CV% of the egg, yolk, eye, optic rudiment, antennae, antennule and caudal papilla's length of *C. nilotica* embryos at 72, 216 and 384 hours

| **Feature** | **Mean length** | **p-value** | **CV%** |
|---|---|---|---|
| egg length at 72 hours | 1.2 | 0.91 | 0.42 |
| egg length at 216 hours | 1.27 | 0.97 | 0.39 |
| egg length at 384 hours | 1.51 | 0.97 | 0.33 |
| yolk length at 72 hours | 1.15 | 0.63 | 0.43 |
| yolk length at 216 hours | 0.64 | 0.89 | 0.78 |
| eye length at 216 hours | 0.1 | 0.95 | 0.55 |
| eye length at 384 hours | 0.14 | 0.78 | 3.57 |
| Optic rudiment length at 72 hours | 0.2 | 0.60 | 2.51 |
| Optic rudiment length at 216 hours | 0.24 | 0.98 | 2.08 |
| Optic rudiment length at 384 hours | 0.33 | 0.93 | 1.51 |
| Antennae length at 72 hours | 0.19 | 0.51 | 2.63 |
| Antennae length at 216 hours | 0.31 | 0.97 | 0.02 |
| Antennae length at 384 hours | 0.71 | 0.95 | 0.7 |
| Antennule length at 72 hours | 0.14 | 0.81 | 3.12 |
| Antennule length at 216 hours | 0.23 | 0.98 | 2.17 |
| Antennule length at 384 hours | 0.6 | 0.94 | 0.83 |
| Caudal papilla length at 216 hours | 0.28 | 0.94 | 1.79 |
| Caudal papilla length at 384 hours | 1.12 | 0.48 | 0.45 |

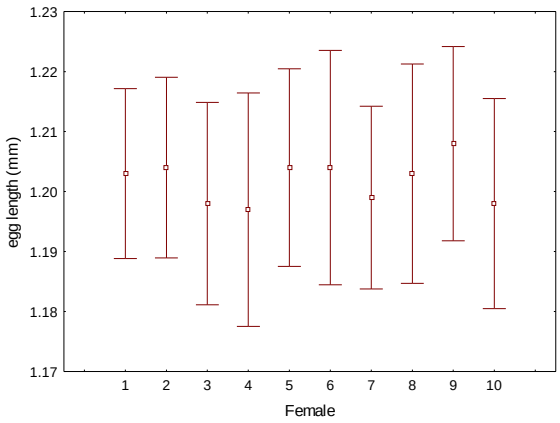


**Fig. 3.23** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the egg length (mm) of embryos from different females of *C. nilotica* at 72 hours

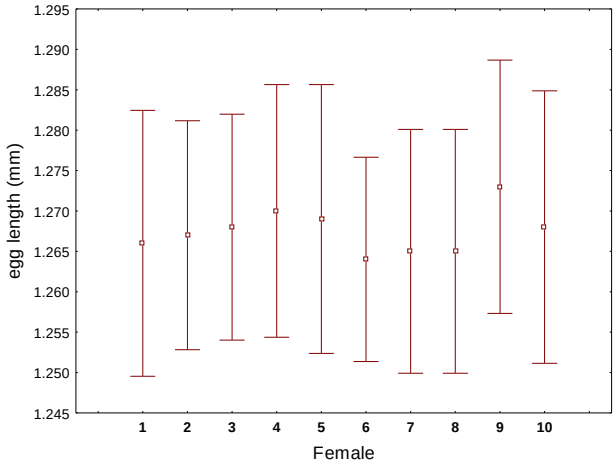


**Fig. 3.24** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the egg length (mm) of embryos from different females of *C. nilotica* at 216 hours

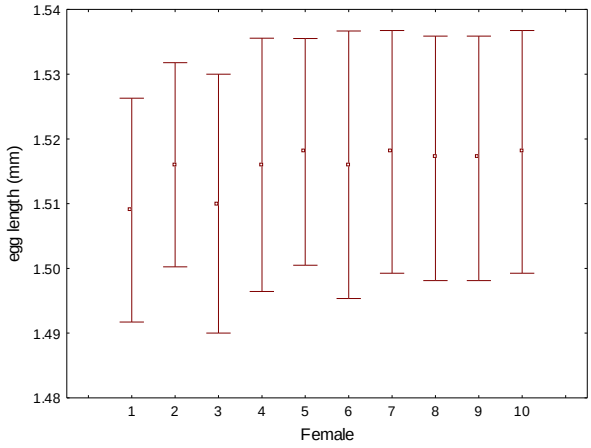


**Fig. 3.25** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the egg length (mm) of embryos from different females of *C. nilotica* at 384 hours

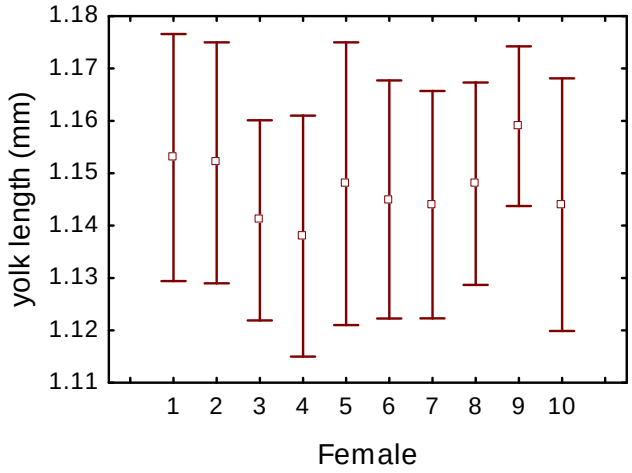


**Fig. 3.26** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the yolk length (mm) of embryos from different females of *C. nilotica* at 72 hours

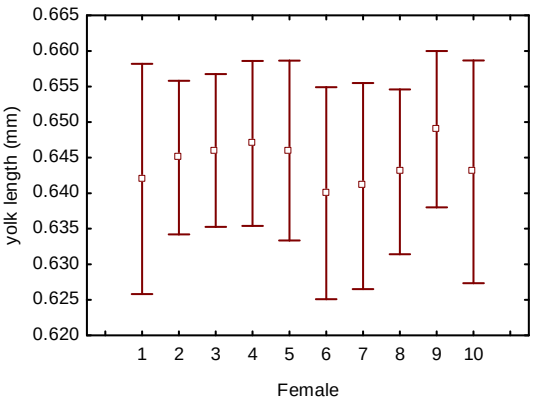


**Fig. 3.27** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the yolk length (mm) of embryos from different females of *C. nilotica* at 216 hours

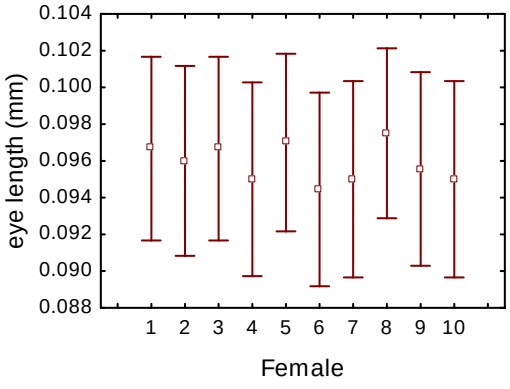


**Fig. 3.28** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the eye length (mm) of embryos from different females of *C. nilotica* at 216 hours

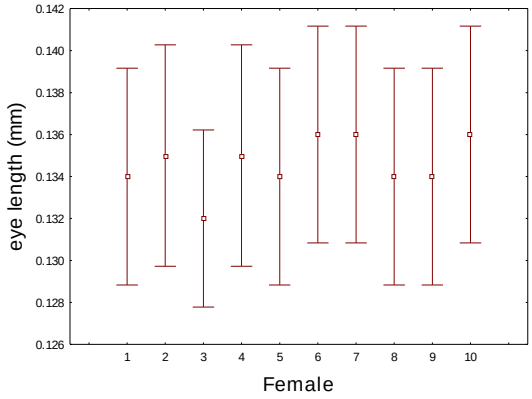


**Fig. 3.29** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the eye length (mm) of embryos from different females of *C. nilotica* at 384 hours

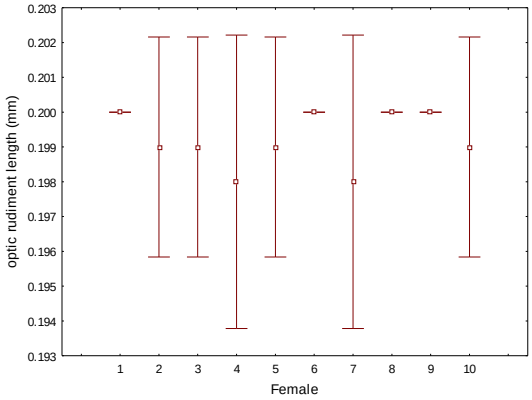


**Fig. 3.30** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the optic rudiment length (mm) of embryos from different females of *C. nilotica* at 72 hours

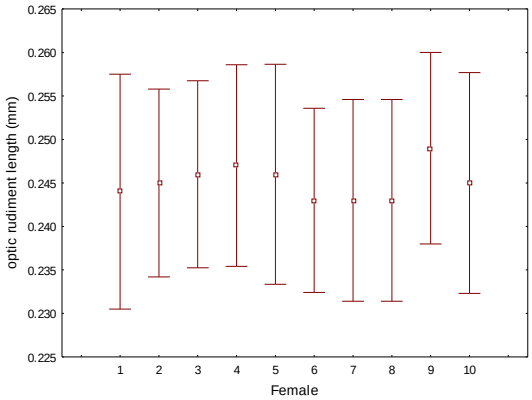


**Fig. 3.31** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the optic rudiment length (mm) of embryos from different females of *C. nilotica* at 216 hours

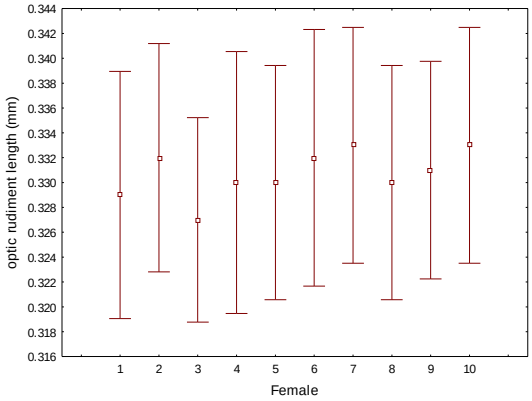


**Fig. 3.32** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the optic rudiment length (mm) of embryos from different females of *C. nilotica* at 384 hours

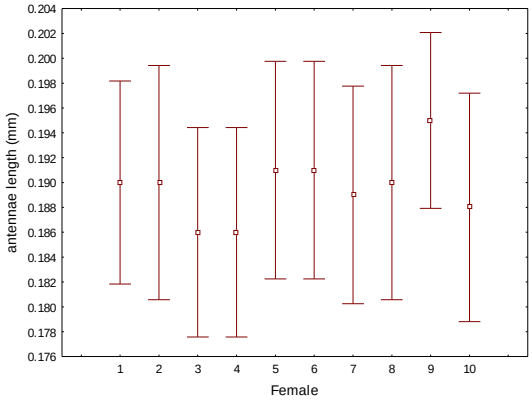


**Fig. 3.33** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the antenna length (mm) of embryos from different females of *C. nilotica* at 72 hours

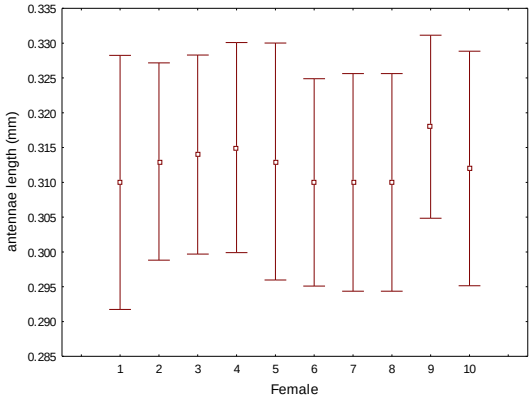


**Fig. 3.34** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the antennae length (mm) of embryos from different females of *C. nilotica* at 216 hours

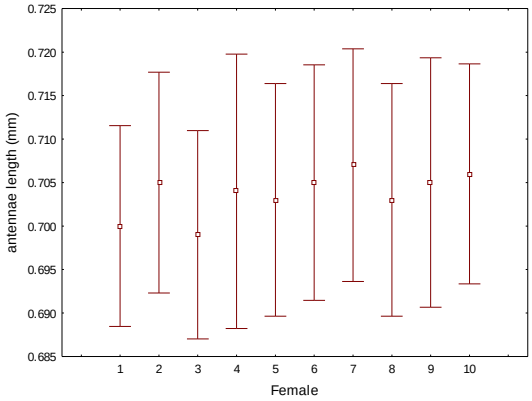


**Fig. 3.35** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the antennae length (mm) of embryos from different females of *C. nilotica* at 384 hours

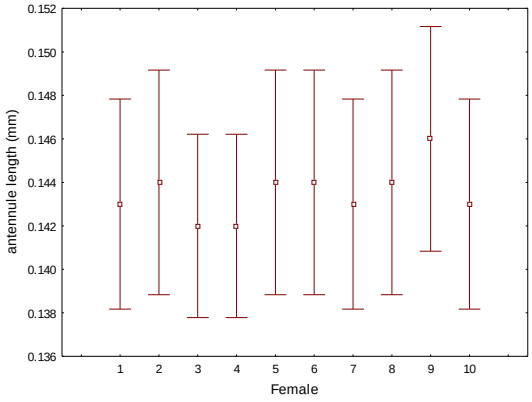


**Fig. 3.36** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the antennule length (mm) of embryos from different females of *C. nilotica* at 72 hours

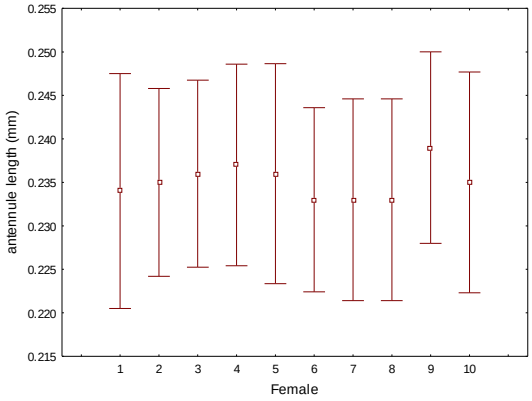


**Fig. 3.37** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the antennule length (mm) of embryos from different females of *C. nilotica* at 216 hours

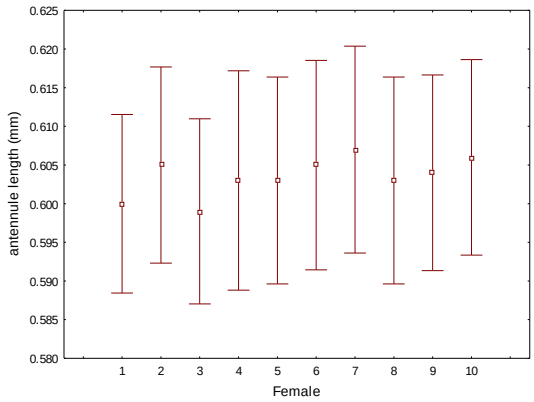


**Fig. 3.38** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the antennule length (mm) of embryos from different females of *C. nilotica* at 384 hours

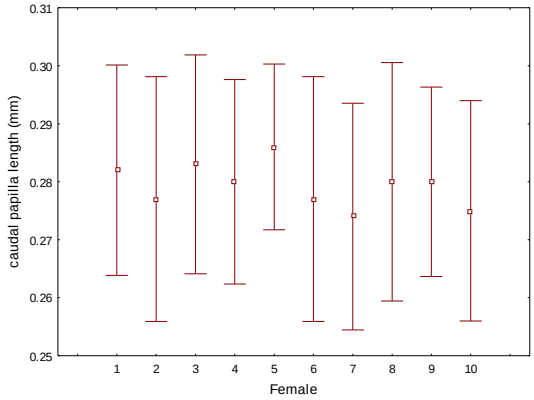


**Fig. 3.39** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the caudal papilla length (mm) of embryos from different females of *C. nilotica* at 216 hours

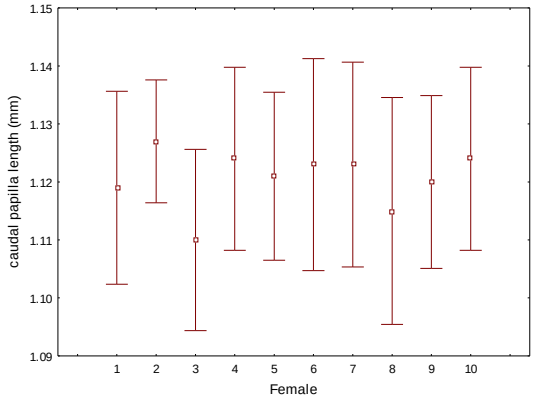


**Fig. 3.40** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the caudal length (mm) of embryos from different females of *C. nilotica* at 384 hours

**Random variability**

From the results of randomly placing eggs from different females into the experimental brood chambers, there seemed to be no differences in mean length, width, length:width ratios and area between mixed eggs. At selected intervals (24, 72, 144, 216, 288 and 336 hours), the length, width, length:width ratios and area of the identified features in the embryonic development were measured. The means of the length, width, length:width ratio and area calculated from the eggs which were randomly mixed were no different from the means calculated from the experiments to assess the intervariability and intravariability of embryonic development (see Appendix A, Figs.A- X).

**3.4 Discussion**

This study allowed morphological characterization of the embryonic structures from the time the eggs were laid until the time they hatched. Females produced between 19 to 60 eggs at spawning (Table 3.1). The embryonic duration of *C. nilotica* from laying to hatching lasted 17 days at 24℃±0.5. This study further demonstrated that embryos developed when they were removed from the brood chambers of parental organisms and incubated in experimental brood chambers. This could be ascribed to the fact that the embryos receive nutrients from the yolk until they hatch, but they need constant aeration (Schram, 1986).

The average measurement of egg size increased from 1.2 x 0.66 mm (at 24hrs) to 2.3 x 0.84 mm (384hrs) (see Table 3.3). It increased from the time of laying until the time they hatched, which is a general occurrence in decapods (Chinnayya, 1974; Lavarias *et al.*, 2002; Nair 1949; Oh and Hartnoll, 2004; Oh *et al.*, 2003; Nazari *et al*., 2000; Saito and Koya, 2001; Thatje *et al.,* 2004; Yamaguchi, 2001). As the embryo develops, the egg membrane alters its permeability allowing water uptake (Kobayashi and Matsuura, 1995; Lavaris *et al.*, 2002). This uptake of water may result in uptake of toxins in the water. This

could be a good reason that the embryo test might be an appropriate and possibly sensitive toxicity test method.

*Caridina nilotica* hatch out in an advanced stage of development and exhibit the direct development seen in decapod crustaceans (Chinnaya, 1974; Lavarias *et al.*, 2002; Müller *et al.,* 2004, Nair, 1949; Nazari *et al.*, 2000). All stages are retained in an embryonic form and every developmental process occurs inside the egg, which means direct development to hatching as a post-embryo, as is also seen in *Caridina laevis* (Nair, 1949), *Paratya australiensis*, (Hancock, 1998) and *Aegla platensis* (Lizardo-Daudt and Bond-Buckup, 2003). During embryonic development there is an intense internal organization of the embryonic structures that are understood mainly as organogenetic processes (Balon, 2001; Nazari, 2000). Given that these processes are occurring internally, the exterior aspect does not change so much, giving the impression that the development is slow (Balon, 2001).

The changes noted from day to day did not occur with the same rate in every specimen. For example, a feature such as development of the eye did not appear at the same time in each specimen. A few eggs might develop an eye a few hours later, which indicates differences in developmental rhythms during embryogenesis as in *Palaemonetes argentinus* (Balon, 2001; Nazari *et al.*, 2000). Variability was kept to a minimum as all the eggs were spawned within 12 hours of one another. This low variability was confirmed by analysis of the results of measurements using ANOVA as there were no significant differences between the sizes of the features of the eggs in either intrafemale, interfemale variability or randomised placing of eggs. The low variability (reflected high p- and low CV% values) is such that it appears as though embryonic development could be useful in the development of the chronic toxicity test method.

In this chapter, seven clearly identifiable ages and the associated developmental features of the embryos were identified as potential toxicity test endpoints (Table 3.4). The embryos will be exposed to reference toxicants to assess the effects of the toxicants on the identified morphometric features.

# CHAPTER 4

## The effects of selected reference toxicants on embryonic development

### 4.1 Introduction

Sodium chloride and sodium sulphate have been identified by Davies and Day (1998) as major inorganic salts involved in salinization of aquatic ecosystems in South Africa. Sodium chloride (NaCl) is linked to natural and agriculturally caused salinization while sodium sulphate ($Na_2SO_4$) is linked to mining and other industrially caused salinization (Scherman *et al*., 2003). In a study by Kefford *et al.* (2002) it was shown that although salts are natural physiological stressors, organisms exposed to salts at a high concentration show classic toxicant concentration – response curves. This is also evident in the database of toxicological tests run by the UCEWQ using NaCl and $Na_2SO_4$ (Palmer *et al.*, 2004), as much of the concentration response data for salts fit the routinely used toxicological analysis models such as Probit. Cadmium chloride ($CdCl_2$) is an important reference toxicant used by the South African Proficiency Test Scheme (for *Daphnia pulex*), of which the UCEWQ is a participant (Muller, pers. comm.).

Salts commonly found in freshwater systems, in decreasing order of toxicity, are magnesium sulphate, sodium sulphate, calcium chloride and sodium chloride (Jooste and Rossouw, 2002). Although the South African Aquatic Ecosystem Water Quality Guidelines do not currently treat salts as toxicants (DWAF, 1996), the current ecological Reserve methods for assessing present state for water quality and determining resource quality do (Jooste and Rossouw, 2002). The concentrations defining the boundaries between different levels of ecosystem protection (ecological categories and management classes) are referred to as ecospecs. Ecospecs for inorganic salts have been determined for each of the ecological categories and management classes using toxicity test results (Jooste and Rossouw, 2002). There is considerable uncertainty about the effect of the increase of salts on aquatic biota and detailed investigations of salinity tolerance are therefore needed.

The UCEWQ database contains acute and short-term chronic toxicity data for a range of indigenous aquatic invertebrates exposed to a range of toxicants (Scherman *et al*., 2002; Palmer *et al*., 2004). The short-term chronic toxicity tests in the UCEWQ toxicity database measure survival and have exposure duration of 10 days. The database was examined for data to the selected inorganic salt (NaCl and $Na_2SO_4$) in which the test species was *Caridina nilotica*.

Table 4.1 shows the test results for *C. nilotica* adults and organisms which were less than 7 days old, from both wild-caught populations (Mpisini stream, KwaZulu Natal) and laboratory-reared populations. The chemicals used were NaCl, $Na_2SO_4$ and $CdCl_2$. Having mortality results for adults and neonates provides no indication of potential effects on (possibly) sensitive early life- history stages such as embryonic development, thus providing motivation for this study. Table 4.2 shows the partial life-cycle test results of No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values for NaCl and $Na_2SO_4$ for *C. nilotica* from Slaughter (2005). The growth was determined by measuring the carapace length and reproduction was determined by the number of eggs produced. The NOEC is determined by Analysis of Variance statistical techniques and is the highest concentration within a toxicity test concentration range that does not have a significant adverse effect on the test organisms when compared to the control (Rand, 1995). The data provided in Tables 4.1 and 4.2 were used to select toxicant test concentrations for NaCl, $Na_2SO_4$ and $CdCl_2$. The highest concentration was selected to be less than the LC50 except for $CdCl_2$. As no chronic toxicity data were available for $CdCl_2$, it was necessary to undertake a range-finding test using *C. nilotica*.

Table 4.1 Selected data from the UCEWQ database: the results reported show NaCl, $Na_2SO_4$ and $CdCl_2$ toxicity to *Caridina nilotica* juveniles. Results reported provide experimental details (organism source (Mpisini stream or LC), exposure mode (R or S), and exposure duration) and experimental endpoints ($LC_{50}$). Experimental endpoints are expressed as mg/L nominal concentration per toxicant (with lower and upper confidence limits, LCL and UCL, respectively). All experiments were undertaken using dechlorinated tap water as diluent with the test endpoint being mortality. (LC- Laboratory culture, R- Rercirculating, S- static)

| Toxicant | River system | Mode of exposure | Test duration | LC50 (mg/L) | LCL (mg/L) | UCL (mg/L) |
|---|---|---|---|---|---|---|
| **$Na_2SO_4$** | Mpisini | R | 240 (h) | 1752 | 1522 | 2006 |
| **$Na_2SO_4$** | Mpisini | R | 240 (h) | 1765 | 1492 | 2088 |
| **$Na_2SO_4$** | LC | R | 240 (h) | 3149 | 2755 | 3511 |
| **$Na_2SO_4$** | LC | R | 240 (h) | 3249 | 2349 | 3685 |
| **$Na_2SO_4$** | LC | S | 48 (h) | 5989 | 4874 | 7181 |
| **$Na_2SO_4$** | LC | S | 48 (h) | 7002 | 4710 | 9024 |
| **$Na_2SO_4$** | LC | S | 48 (h) | 5734 | 5084 | 6614 |
| **$Na_2SO_4$** | LC | S | 48 (h) | 5477 | 4840 | 6007 |
| **NaCl** | LC | S | 48 (h) | 5979 | 4823 | 7059 |
| **NaCl** | LC | S | 48 (h) | 5955 | 5100 | 6752 |
| **NaCl** | LC | S | 48 (h) | 4450 | 3709 | 5196 |
| **NaCl** | LC | S | 48 (h) | 5487 | 4528 | 6446 |
| **$CdCl_2$** | LC | S | 48 (h) | 0.05 | 0.02 | 0.08 |
| **$CdCl_2$** | LC | S | 48 (h) | 0.07 | 0.04 | 0.1 |
| **$CdCl_2$** | LC | S | 48 (h) | 0.09 | 0.04 | 0.14 |

Table 4.2 The LOEC and NOEC values for NaCl and $Na_2SO_4$ on the growth and reproduction of *C. nilotica* (Slaughter, 2005)

| Salt | Endpoint | LOEC (mg/L) | NOEC (mg/L) |
|---|---|---|---|
| **NaCl** | Mortality | 2700 | 1900 |
| | Growth | 2200 | 1900 |
| | Reproduction | 2700 | 1900 |
| **$Na_2SO_4$** | Mortality | 2530 | 1900 |
| | Growth | 2530 | 1900 |
| | Reproduction | 2530 | 1900 |

The results from chapter 3 identified 7 developmental stages. The aim of chapter 4 therefore is to assess whether *C. nilotica* embryonic development can be used as experimental endpoints in toxicity tests by determining the effects of the selected reference toxicants on embryonic development. This was done by assessing the effects of three toxicants (NaCl, $Na_2SO_4$ and $CdCl_2$) on the

following features of the developing embryo: size, rate of development and mortality of developing embryos.

## 4.2 Materials and methods

In order to obtain embryos of known ages, eggs were removed from gravid females which had been gravid (females carrying extruded eggs) for less than 24 hours; the eggs had to be newly spawned to follow the embryology from spawning until hatching. Gravid females were collected from all the culture tanks in the afternoon prior to commencement of each test and the gravid females collected early the following day would have eggs less than 24 hours old. The newly spawned eggs were then extracted from the females using a soft paintbrush, and eggs were randomly distributed to experimental brood chambers.

### 4.2.1 Experimental design

Five concentrations of each of the toxicants were prepared. The control was dechlorinated tap water; each concentration was run in three replicates to assess variability in embryo development (Mackay *et al.* in Rand, 1995). Various water quality parameters were measured in each chamber. Temperature and conductivity were measured every day using a thermometer and a conductivity meter respectively, while pH and dissolved oxygen were measured using the pH and DO meters respectively, every third day. Exposure solutions were also changed every 3 days to prevent fungal growth on the developing eggs. All experiments were undertaken at a temperature of 24ºC±0.5ºC.

When the embryonic development of *C. nilotica* was determined (Chapter 3), numerous morphological features were identified. However, seven ages were identified as possible sub-lethal endpoint measures, because the morphological features at these ages were easily identified and could be measured (Chapter 3, Figs. 3.1-3.6). The age of development at specified ages of eggs was noted and the sizes of the various appendages were measured (length, width, length:width ratio and area). The developmental ages are represented in Table 4.3.

Table 4.3 The ages which were identified as potential experimental toxicity endpoints. The ages refer to the age of the embryo when the specified developmental features could be distinguished

| **Age (hrs)** | **Feature** |
|---|---|
| 24 | egg and yolk |
| 72 | antennae, antennules and optic rudiment |
| 144 | caudal papilla |
| 216 | eye and carapace |
| 288 | heartbeat and pleopods |
| 336 | development of all features |
| 408 | hatching |

Since there were 7 developmental ages to monitor, and each required a sample size of 10 eggs, a total of 70 eggs was required for each brood chamber. At the predetermined developmental stages, 10 eggs were removed from each chamber and placed in a Petri dish with dechlorinated tap water and the live specimens were examined under the dissecting microscope (12 x magnification). They were then fixed in 4% formalin and the developmental stages were photographed using an Olympus 4040 Zoom digital camera (Olympus American Inc., USA) attached to a light microscope and the images saved to a computer. The identified features, mortality and abnormalities were recorded, and morphological measures undertaken using AnalySIS by Soft Imaging Systems (SIS).

### 4.2.2 Exposure concentrations

The concentrations provided in Tables 4.1 and 4.2 guided the choice of concentrations used in this study. Nominal exposure concentrations were used to calculate the NOEC and LOEC values.

**Sodium sulphate**

The concentrations chosen were 1000mg/L, 2000mg/L, 3000mg/L, 4000mg/L and 5000mg/L and a control using dechlorinated tap water.

**Sodium chloride**

The following concentrations were chosen: 1000mg/L, 2000mg/L, 3000mg/L, 4000mg/L and 6000 mg/L and a control using dechlorinated tap water.

**Cadmium chloride**

The cadmium chloride ($CdCl_2$) was in a stock solution of 10mg/L as provided by the Proficiency Test Scheme for *Daphnia pulex* (de Kock, pers. comm.). The concentrations were selected based on the results of acute and chronic tests from the UCEWQ and USEPA databases (http://www.epa.gov/ecotox/ (17/10/2005) for *C. nilotica* and *D. pulex* respectively. Selected test concentrations of 0.31mg/L, 0.63mg/L, 1,25mg/L, 2,5mg/L and 5mg/L were prepared by serial dilutions of the standard 10mg/L stock solution (Rand, 1995).

### 4.2.3 Statistical Analysis

A one-way Analysis of Variance (ANOVA) test was used to undertake comparisons of features between embryos in the control and the different concentrations (StatSoft, 2002). The results for all three replicates within each experiment were analysed using ANOVA in Statistica Version 6 (StatSoft, 2002), followed by a parametric post-hoc test (Tukey honest significant difference (HSD)) to determine which concentrations were statistically different from the control. The Lowest Observed Effect Concentration (LOEC) was determined as the lowest concentration tested that was statistically significantly different in one of length, width, length:width ratio or area of the measured feature relative to the control using Tukey HSD test. The No Observed Effect Concentration (NOEC) was determined as the highest concentration tested that did not result in a

statistically significant different effect in length, width, length:width ratio and area of all features relative to the control.

### 4.3 Results

The pH ranged from 6.2 to7.6 and the DO ranged from 7.5 mg/L to 8.3mg/L. Differences in length, width, length:width ratio and area of eggs between the concentrations were tested and the results showed that there were no significant differences between the replicates. Since there were no differences between the replicates, data from the replicates were combined. There were significant differences between concentrations over time for length, width, length: width ratios and area. However, as the same trends were displayed for each of the measured features, only the results for length are graphically represented in this chapter, while graphs for width, length:width ratios and area are reported in Appendix B (Figs. A-N)

#### 4.3.1 Sodium sulphate

Eggs hatched only in the control and the lowest test concentration of 1000mg/L. The controls in all replicates had 0% mortality, while all the other concentrations the eggs did not hatch. Mortality started at 24 hours in the concentration of 5000mg/L. By 336 hours, there was 100% mortality of eggs at 4000mg/L and 5000mgL and, at 408 hours (where hatching occurred in the control and 1000mg/L), there was 100% mortality of eggs at 2000mg/L and 3000mg/L as well (Fig. 4.1). At 1000mg/L, 89% of the eggs hatched at 408 hours and all the eggs in the control hatched (Fig. 4.2).

From the time the experiment commenced until 72 hours there was an increase in length, width and area of egg in all concentrations. These increases were not significantly different from the control and between concentrations (Figs. 4.3 and 4.4). At 144 hours, the time when the caudal papilla appeared in the control, the first observed differences in the length, width and area of all the features were

noted. These differences were statistically significantly different when compared to the control for each of the concentrations, except at 1000mg/L for all features ($p>0.05$) and at 2000mg/L for egg length, antennae length and antennule length (Figs. 4.5-4.9). By this time (144 hours), there was 7% mortality in 3000mg/L, 11% mortality in 4000mg/L and 15% mortality in 5000mg/L (Fig. 4.2).

At 216 hours, the eye was not visible as expected at 2000mg/l, 3000mg/l, 4000mg/L and 5000mg/L. However, in the eggs in the control and those at 1000mg/L, the eye was visible. At 288 hours the palpitations of the heart can be seen at 1000mg/L, 2000mg/L and 3000mg/L, but not at 4000mg/l and 5000mg/L. At 336 hours at 1000mg/L, 2000mg/L and 3000mg/L all the features were developed. The length, width and area were smaller and statistically significantly different from the control except at 1000mg/L. The LOEC value at 144 hours was 2000mg/L, the NOEC value was 1000mg/L (Table 4.4).

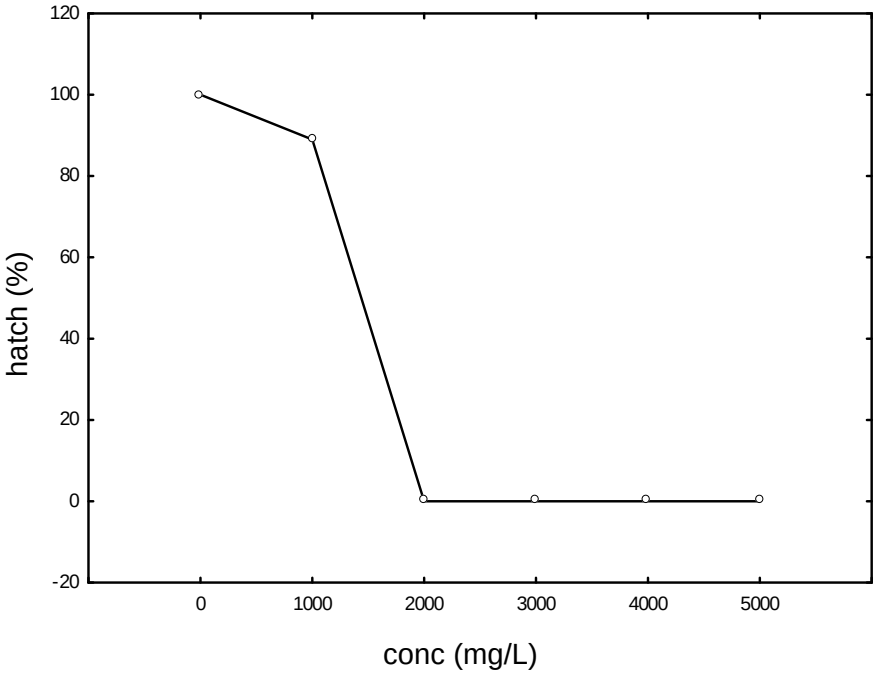


**Fig. 4.1** Total hatching success (%) of *C. nilotica* embryos after 408 hours exposure to $Na_2SO_4$ at different concentrations

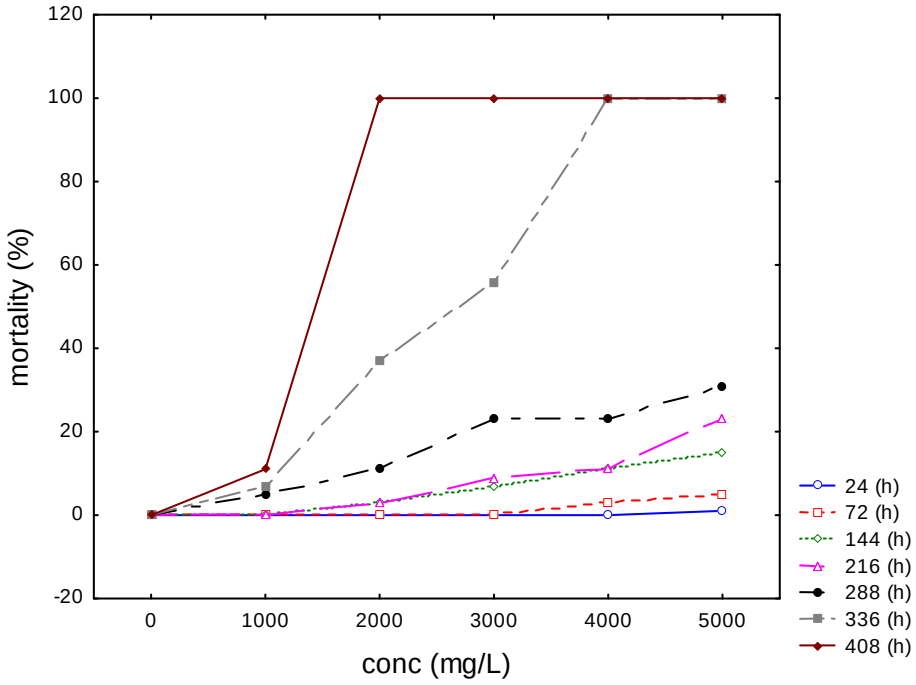


**Fig. 4.2** Total mortality (%) of *C. nilotica* embryos exposed to increasing $Na_2SO_4$ concentrations. Each line represents a different exposure period. Data for the replicates were combined for each concentration

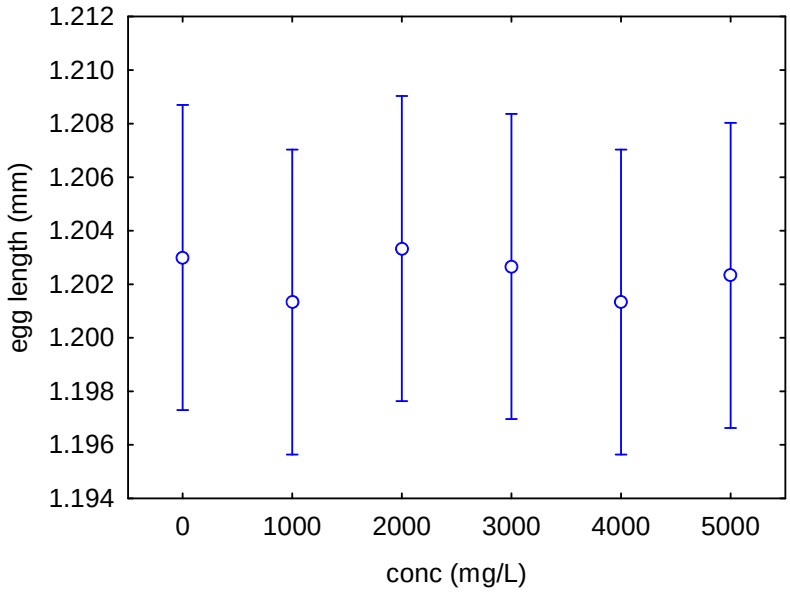


**Fig. 4.3** Mean (o) and 95% confidence intervals (I) of egg length (mm) of *C. nilotica* embryos at 72 hours. The egg lengths are not statistically significantly different

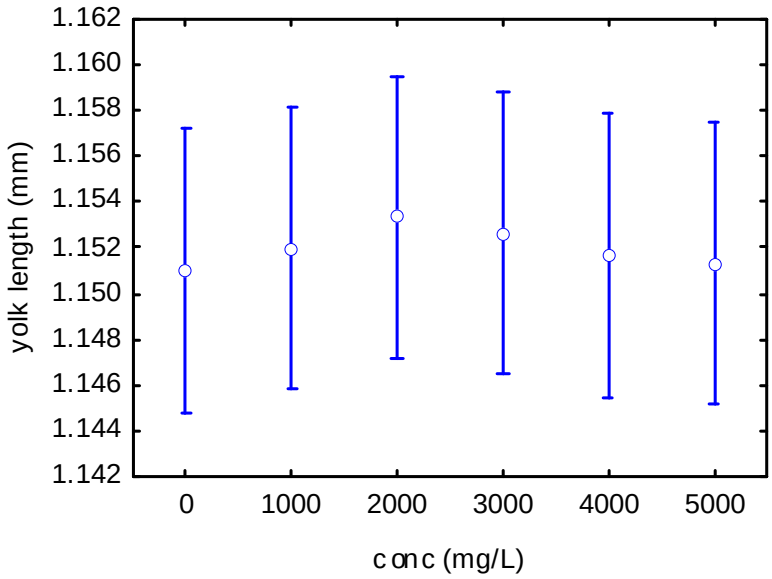


**Fig. 4.4** Mean (o) and 95% confidence intervals (I) of yolk length (mm) of *C. nilotica* embryos at 72 hours. The yolk lengths are not statistically significantly different

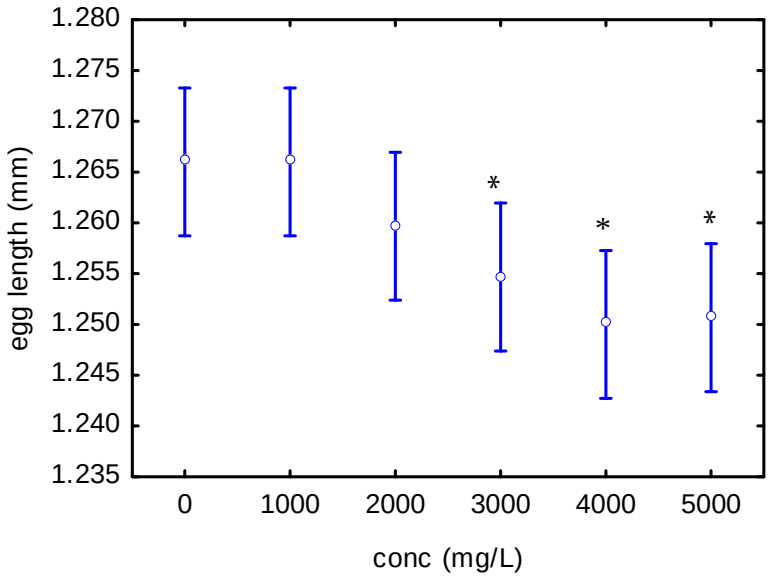


**Fig. 4.5** Mean (o) and 95% confidence intervals (I) of egg length (mm) of *C. nilotica* embryos at 144 hours. Eggs were significantly smaller at and above $Na_2SO_4$ concentrations of 3000mg/L than at 200mg/L and below. The * indicates the concentrations where the egg length was significantly different from the control

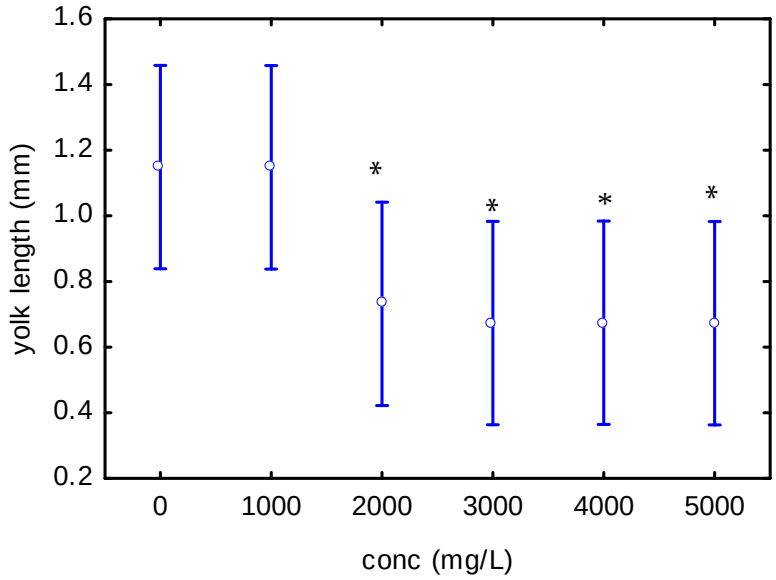


**Fig. 4.6** Mean (o) and 95% confidence intervals (I) of yolk length (mm) of *C. nilotica* embryos at 144 hours. Yolks were significantly smaller at and above $Na_2SO_4$ concentrations of 2000mg/L than at 100mg/L and below. The * indicates the concentrations where the yolk length was significantly different from the control

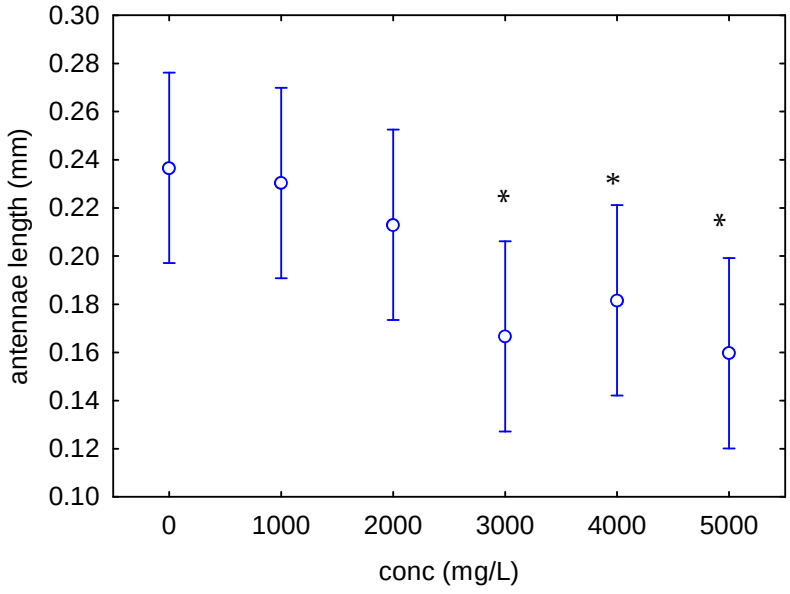


**Fig. 4.7** Mean (o) and 95% confidence intervals (I) of antennal length (mm) of *C. nilotica* embryos at 144 hours. Antennaes were significantly smaller at and above $Na_2SO_4$ concentrations of 3000mg/L than at 200mg/L and below. The * indicates the concentrations where the antennae length was significantly different from the control

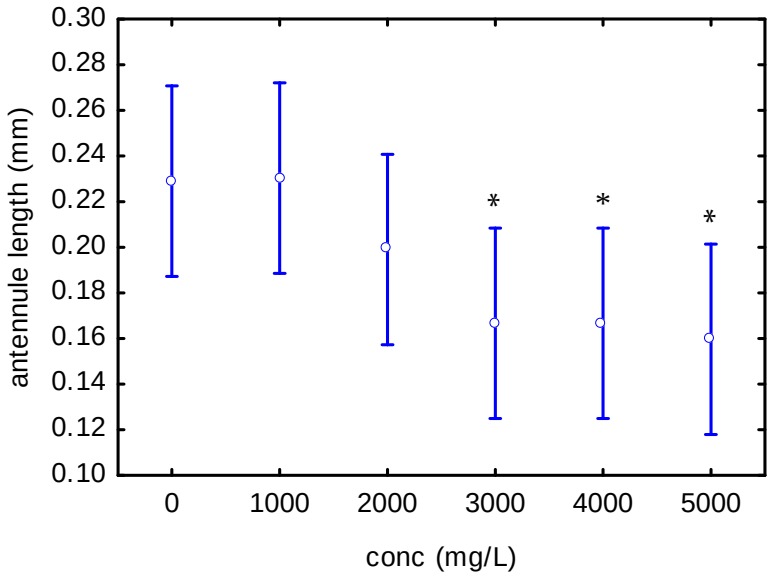


**Fig. 4.8** Mean (o) and 95% confidence intervals (Ī) of antennule length (mm) of *C. nilotica* embryos at 144 hours. Antennules were significantly smaller at and above $Na_2SO_4$ concentrations of 3000mg/L than at 200mg/L and below. The * indicates the concentrations where the antennule length was significantly different from the control

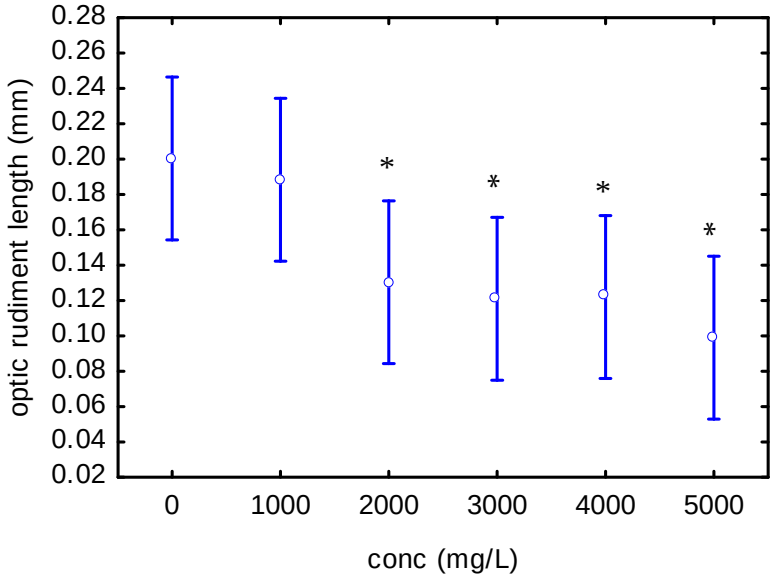


**Fig. 4.9** Mean (o) and 95% confidence intervals (Ī) of optic rudiment length (mm) of *C. nilotica* embryos at 144 hours. Optic rudiments were significantly smaller at and above $Na_2SO_4$ concentrations of 2000mg/L than at 100mg/L and below. The * indicates the concentrations where the optic rudiment length was significantly different from the control

### 4.3.2 Sodium chloride

All control replicates had 0% mortality and 100% hatching success. At 1000mg/L, 93% of the eggs hatched, in 2000mg/L 49% of the eggs hatched and in 3000mg/L 23% of the eggs hatched (Fig. 4.10). There was mortality in 4000mg/L concentration of NaCl within 72 hours. At 408 hours there was 100 % mortality at 4000mg/L and 6000mg/L (Fig. 4.11).

The length, width and area of the egg, yolk, antennule, antennae and optic rudiment, increased gradually in all concentrations until 144 hours. The increases were not significantly different from the control and between concentrations (Figs. 4.12-4.16). At 144 hours the appearance of the caudal papilla was observed as expected in all concentrations. From 3000mg/L to 6000mg/L at 216 hours, where the eye developed, the length, width and area of the features were smaller when compared with the controls (Figs. 4.17- 4.20). At 288 hours the heart palpitations were seen at 1000mg/l and 2000mg/L, and the measured features continued to be smaller in length, width and area when compared to the control (Fig. 4.21-4.25). At 336 hours all the developmental features can be observed in all the concentrations but at 3000mg/L to 6000mg/L the features were significantly smaller when compared to the control.

The LOEC observed was at 3000mg/l based on the lowest concentration which had a statistically different effect from the control and the NOEC was at 2000mg/l based on the highest concentration which did not have a statistically different effect from the control (Table 4.4). The length of the egg, yolk, antennae, antennule and optic rudiment at 216 hours were the morphological features which allowed the identification of the LOEC/NOEC values.

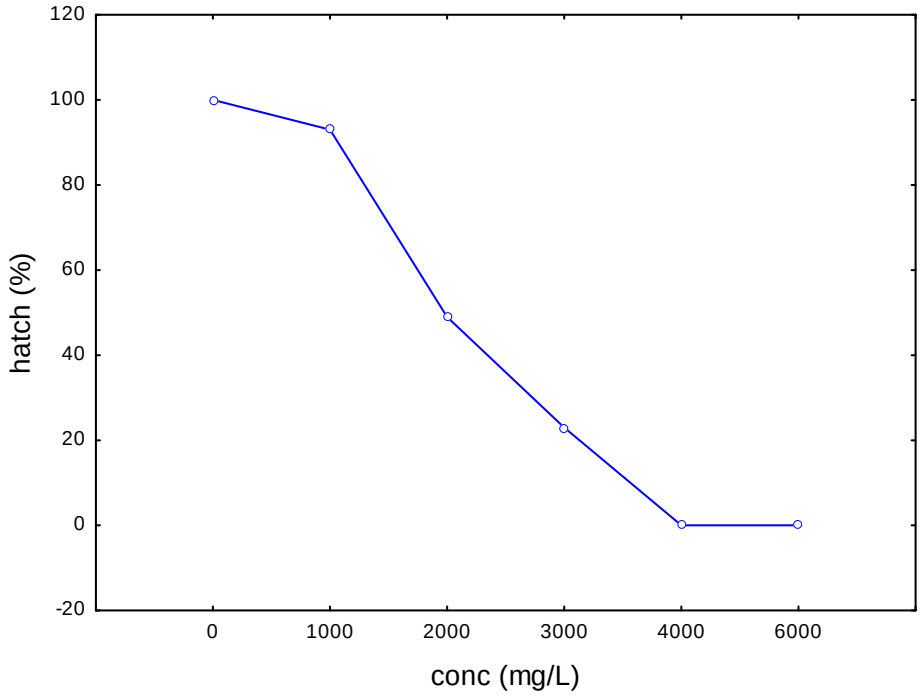


**Fig. 4.10** Total hatching success (%) of *C. nilotica* embryos after 408 hours exposure to NaCl at different concentrations

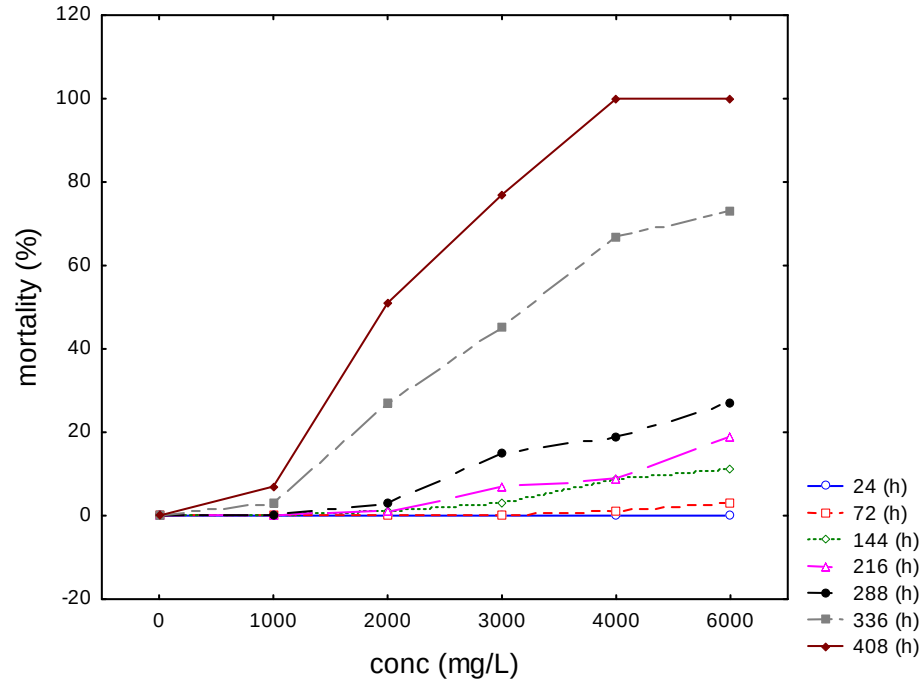


**Fig. 4.11** Total mortality (%) of *C. nilotica* embryos exposed to increasing NaCl concentrations. Each line represents a different exposure period. Data for the replicates were combined for each concentration

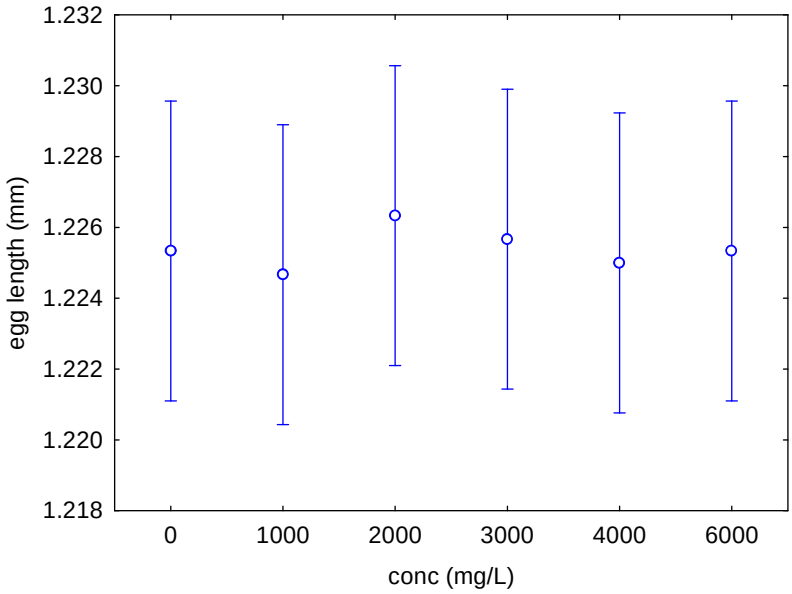


**Fig. 4.12** Mean (o) and 95% confidence intervals (I) of egg length (mm) of *C. nilotica* embryos at 144 hours. The egg lengths are not statistically significantly different

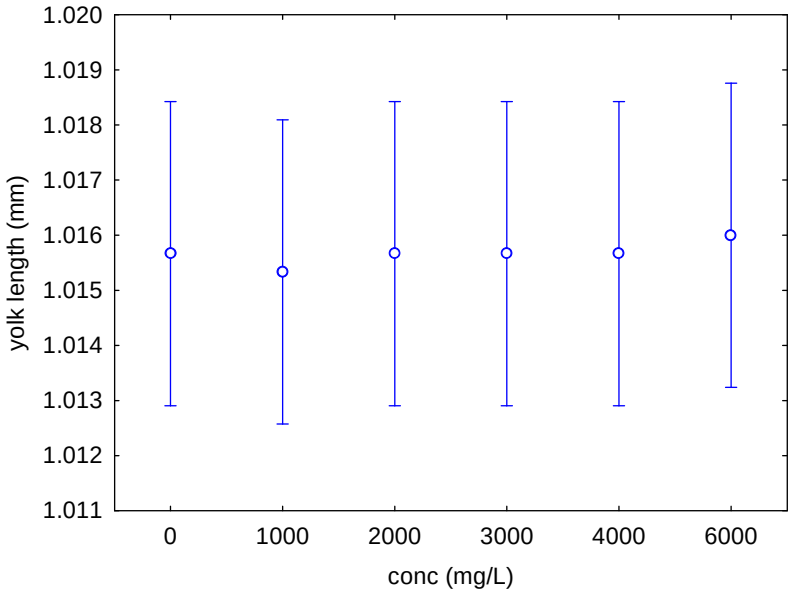


**Fig. 4.13** Mean (o) and 95% confidence intervals (I) of yolk length (mm) of *C. nilotica* embryos at 144 hours. The yolk lengths are not statistically significantly different

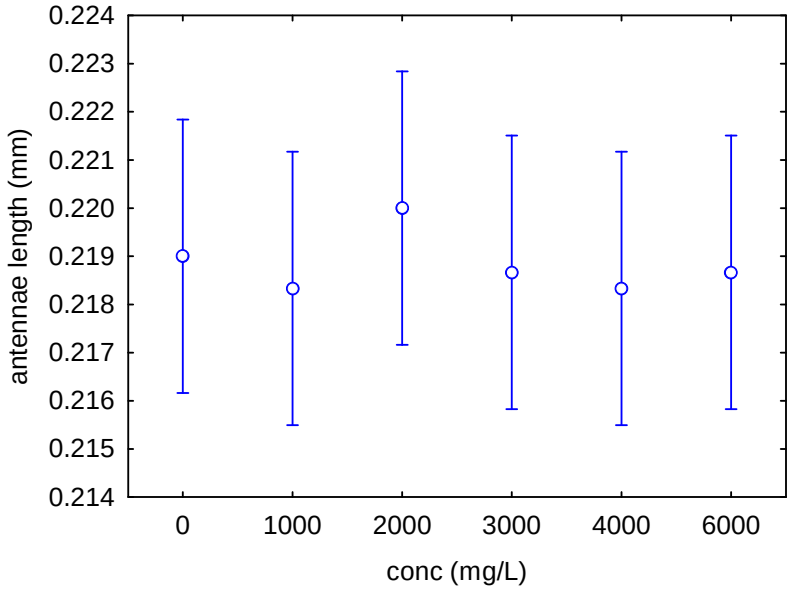


**Fig. 4.14** Mean (o) and 95% confidence intervals (I) of antennal length (mm) of *C. nilotica* embryos at 144 hours. The antennal lengths are not statistically significantly different

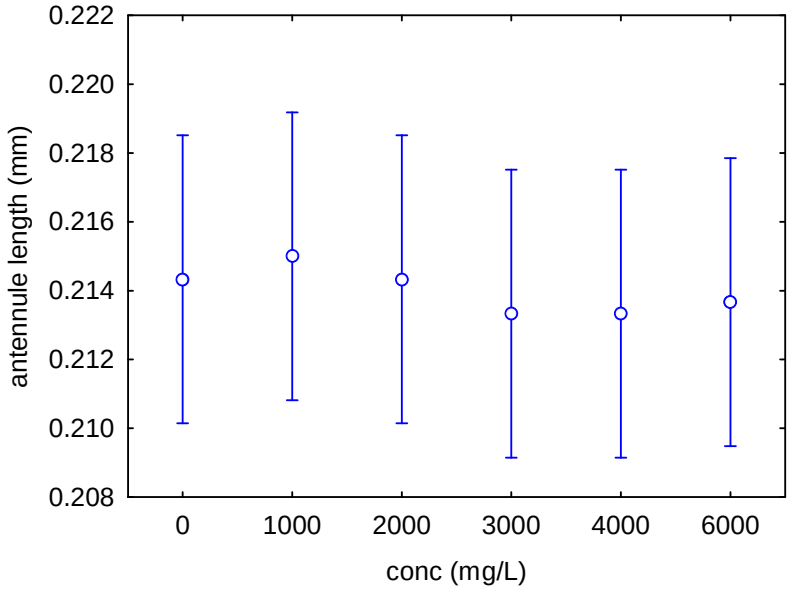


**Fig. 4.15** Mean (o) and 95% confidence intervals (I) of antennule length (mm) of *C. nilotica* embryos at 144 hours. The antennule lengths are not statistically significantly different

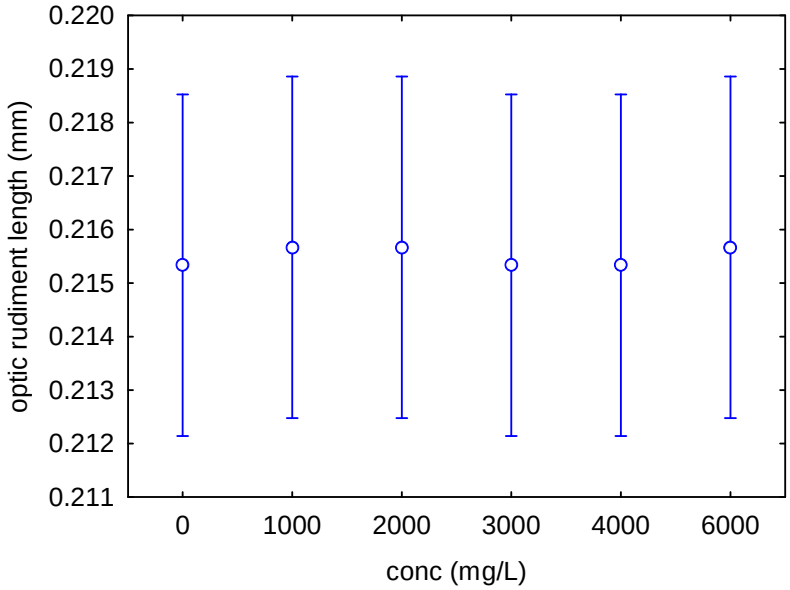


**Fig. 4.16** Mean (o) and 95% confidence intervals (Ī) of optic rudiment length (mm) of *C. nilotica* embryos at 144 hours. The optic rudiment lengths are not statistically significantly different

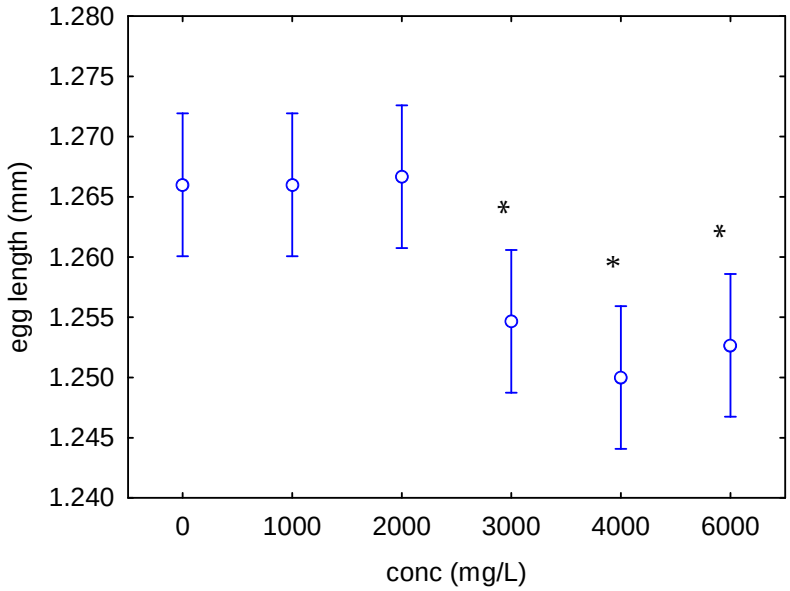


**Fig. 4.17** Mean (o) and 95% confidence intervals (Ī) of egg length (mm) of *C. nilotica* embryos at 216 hours. Eggs were significantly smaller at and above NaCl concentrations of 3000mg/L than at 200mg/L and below. The * indicates the concentrations where the egg length was significantly different from the control

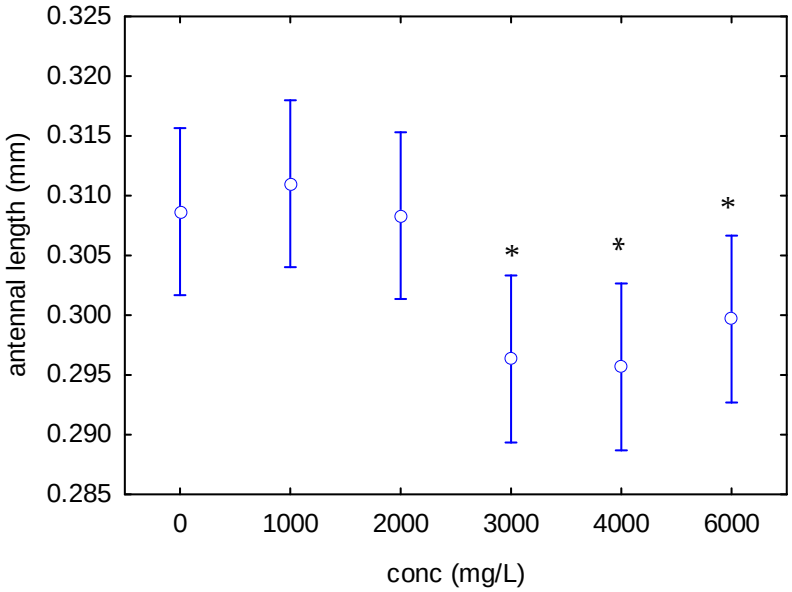


**Fig. 4.18** Mean (o) and 95% confidence intervals (|) of antennal length (mm) of *C. nilotica* embryos at 216 hours. Antennaes were significantly smaller at and above NaCl concentrations of 3000mg/L than at 200mg/L and below. The * indicates the concentrations where the antennal length was significantly different from the control

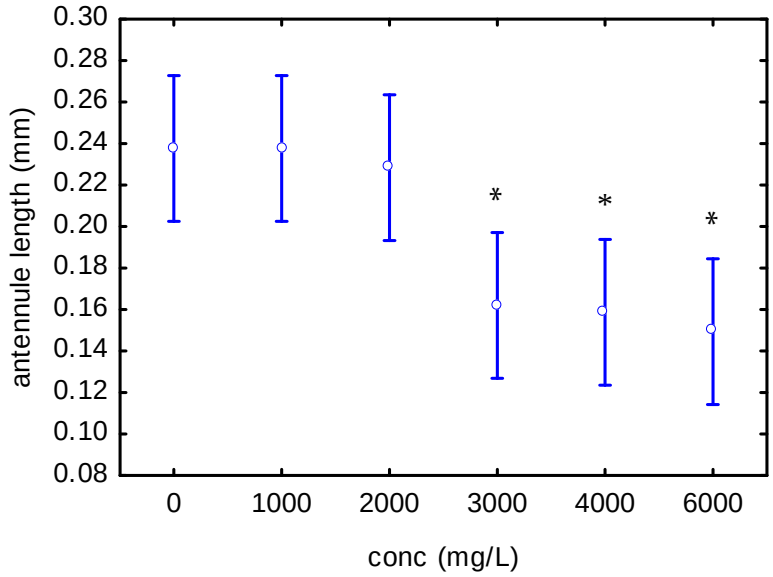


**Fig. 4.19** Mean (o) and 95% confidence intervals (|) of antennule length (mm) of *C. nilotica* embryos at 216 hours. Antennules were significantly smaller at and above NaCl concentrations of 3000mg/L than at 200mg/L and below. The * indicates the concentrations where the antennule length was significantly different from the control

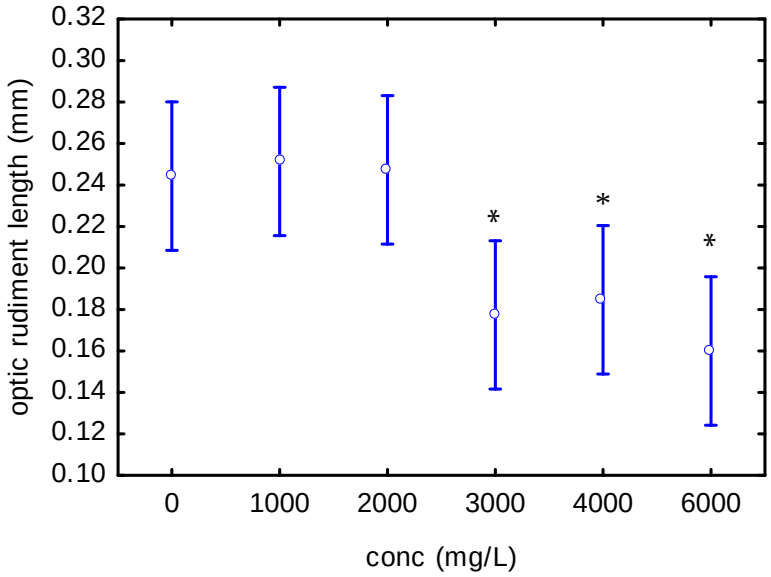


**Fig. 4.20** Mean (o) and 95% confidence intervals (I) of optic rudiment length (mm) of *C. nilotica* embryos at 216 hours. Optic rudiments were significantly smaller at and above NaCl concentrations of 3000mg/L than at 200mg/L and below. The * indicates the concentrations where the optic rudiment length was significantly different from the control

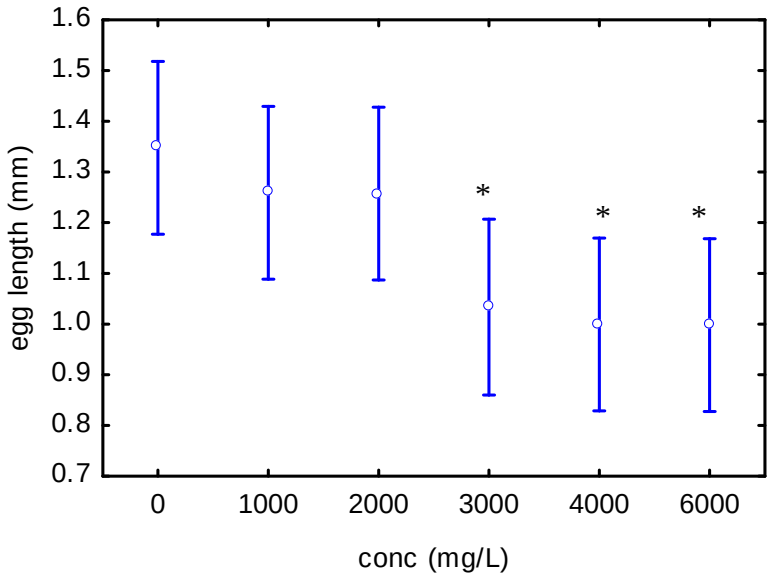


**Fig. 4.21** Mean (o) and 95% confidence intervals (I) of egg length (mm) of *C. nilotica* embryos at 216 hours. Eggs were significantly smaller at and above NaCl concentrations of 3000mg/L than at 200mg/L and below. The * indicates the concentrations where the egg length was significantly different from the control

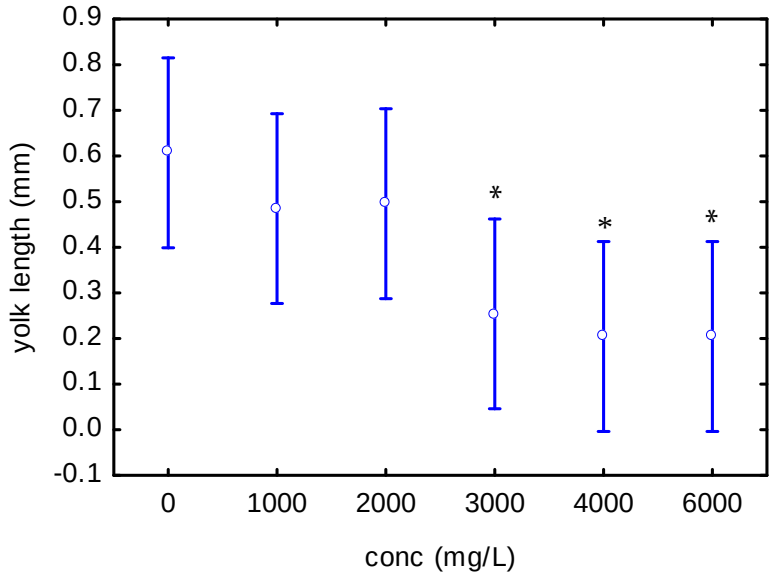


**Fig. 4.22** Mean (o) and 95% confidence intervals ( ) of yolk length (mm) of *C. nilotica* embryos at 288 hours. Yolks were significantly smaller at and above NaCl concentrations of 3000mg/L than at 200mg/L and below. The * indicates the concentrations where the yolk length was significantly different from the control

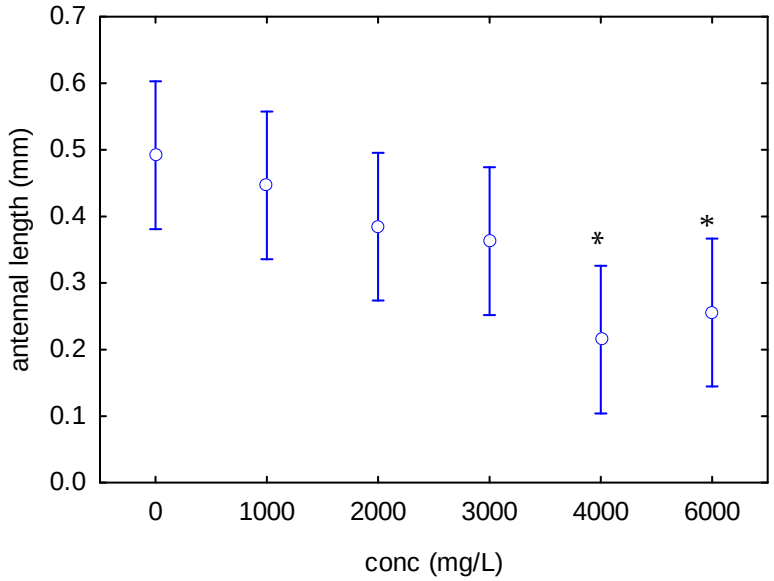


**Fig. 4.23** Mean (o) and 95% confidence intervals ( ) of antennal length (mm) of *C. nilotica* embryos at 288 hours. Antennaes were significantly smaller at and above NaCl concentrations of 4000mg/L than at 300mg/L and below. The * indicates the concentrations where the antennal length was significantly different from the control

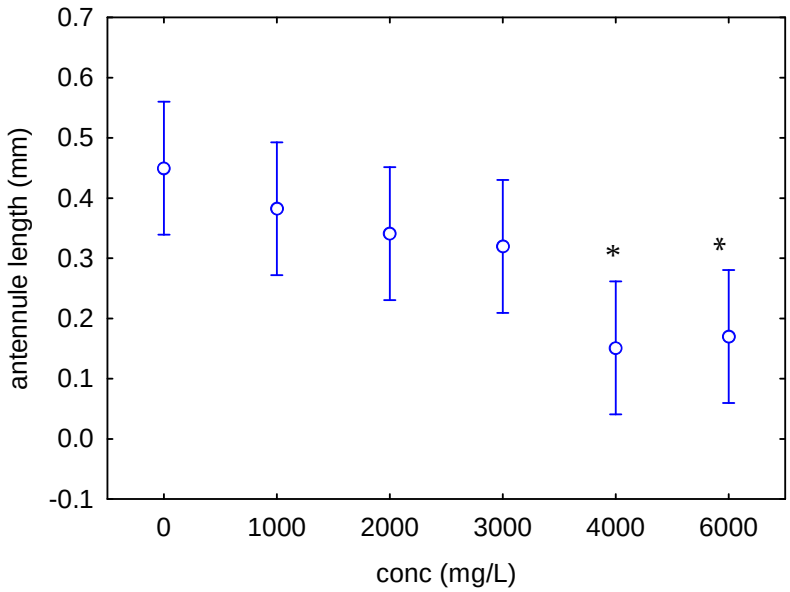


**Fig. 4.24** Mean (o) and 95% confidence intervals ( ) of antennule length (mm) of *C. nilotica* embryos at 288 hours. Antennules were significantly smaller at and above NaCl concentrations of 4000mg/L than at 300mg/L and below. The * indicates the concentrations where the antennule length was significantly different from the control

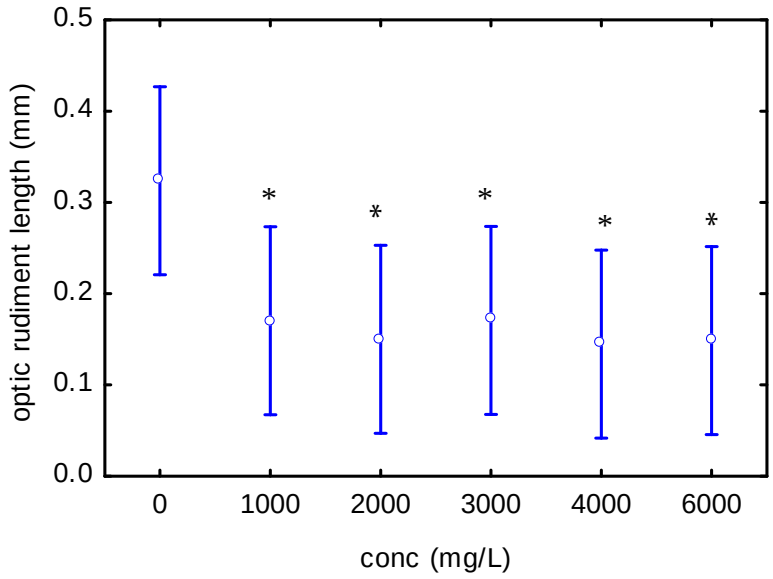


**Fig. 4.25** Mean (o) and 95% confidence intervals ( ) of optic rudiment length (mm) of *C. nilotica* embryos at 288 hours. Optic rudiments were significantly smaller at and above NaCl concentrations of 1000mg/L. The * indicates the concentrations where the optic rudiment length was significantly different from the control

### 4.3.3 Cadmium chloride

At 0.31mg/L 13% of the eggs hatched while in the controls all the eggs hatched (Fig. 4.26). At 24 hours mortality was observed in all the concentrations, and at 216 hours there was 100% mortality at 2.5 mg/L and 5mg/L (Fig. 4.27). At 288 hours there was 100% mortality at 1.25mg/L and at 408 hours there was 100% mortality at 0.63mg/L. This strongly suggests that the concentration range was too high.

After 24 hours exposure, a comparison of measured responses with controls showed a significant reduction in length, width and area of the features in all the concentrations. Over time until hatching the length, width and area of the features were smaller when compared to the control. The cadmium chloride proved to be highly toxic because most of the eggs did not reach the hatching stage. There was an insignificant mortality of 3% in the control, the allowed mortality in the control is 10% (Rand, 1995).

The LOEC was at 0.31mg/L at 24 hours, which leads to an estimated NOEC of between 0mg/L and 0.312mg/L of $CdCl_2$ (Table 4.4).

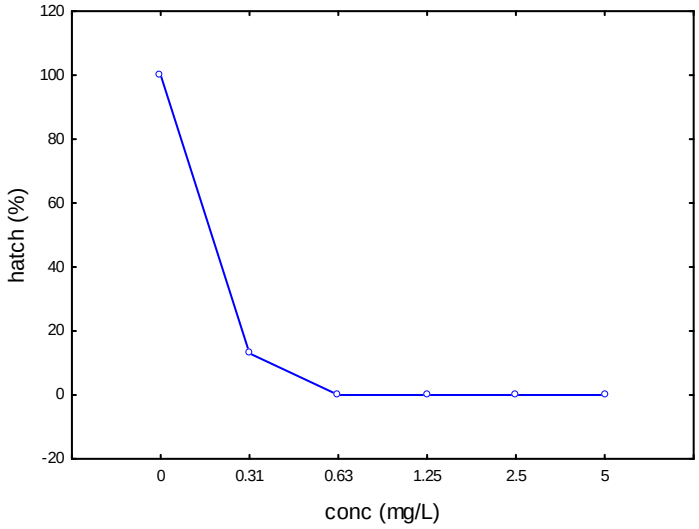


**Fig. 4.26** Total hatching success (%) of *C. nilotica* embryos after 408 hours exposure to $CdCl_2$ at different concentrations

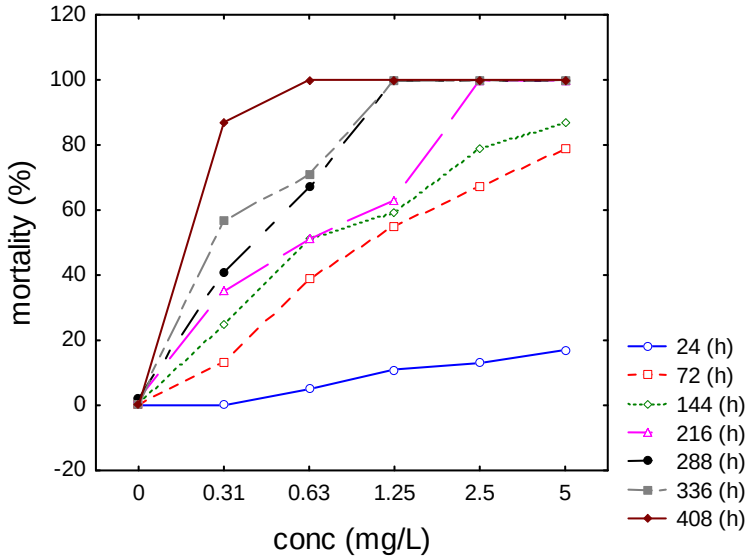


**Fig. 4.27** Total mortality (%) of *C. nilotica* embryos exposed to increasing $CdCl_2$ concentrations. Each line represents a different exposure period. Data for the replicates were combined for each concentration

Table 4.4 The estimated NOEC and LOEC values for NaCl, $Na_2SO_4$ and $CdCl_2$ over time, for the various developmental features of *C. nilotica*.

| **Toxicant** | **Time (hrs)** | **Feature** | **LOEC (mg/L)** | **NOEC(mg/L)** |
|---|---|---|---|---|
| $Na_2SO_4$ | 144 | egg | 3000 | 2000 |
| | | yolk | 2000 | 1000 |
| | | antennae | 3000 | 2000 |
| | | antennule | 3000 | 2000 |
| | | optic rudiment | 2000 | 1000 |
| | 216 | caudal papilla | 3000 | 2000 |
| | 288 | eye | 4000 | 3000 |
| | 336 | pleopods | 3000 | 2000 |
| | | | | |
| NaCl | 216 | egg | 3000 | 2000 |
| | | yolk | 3000 | 2000 |
| | | antennae | 3000 | 2000 |
| | | antennule | 3000 | 2000 |
| | | optic rudiment | 3000 | 2000 |
| | | caudal papilla | 3000 | 2000 |
| | 288 | eye | 4000 | 3000 |
| | 336 | pleopods | 3000 | 2000 |
| | | | | |
| $CdCl_2$ | 24 | egg | 0.63 | 0.31 |
| | | yolk | 0.31 | < 0.31 |
| | 72 | antennae | 0.31 | <0.31 |
| | | antennule | 0.31 | <0.31 |
| | | optic rudiment | 0.31 | <0.31 |
| | 144 | caudal papilla | 0.31 | <0.31 |
| | 216 | eye | 0.31 | <0.31 |
| | 288 | pleopods | | |

## 4.4 Discussion and conclusions

In previous studies the hatching percentage of embryos from egg sacs has been the focus of embryo toxicity studies (Lee and Oshima, 1998, Lee *et al.*, 1996). The toxicity tests undertaken in this study showed that most concentrations of $Na_2SO_4$, NaCl and $CdCl_2$ administered during embryonic development resulted in reduction of growth and increased mortality rate.

NaCl was the least lethal of the 3 toxicants (Table 4.4); the NOEC value for NaCl was the highest. Mortality started at 72 hours in 4000mg/L (Fig. 4.11), where 1%

of the embryos died. NaCl is followed by $Na_2SO_4$ in lethality. At 24 hours there was 1% mortality at 5000mg/L $Na_2SO_4$ (Fig. 4.2) while $CdCl_2$ was most toxic, resulting in 5% mortality at 24 hours at 0.63mg/L (Fig. 4.27). The pattern of $Na_2SO_4$ being more toxic than NaCl was evident in a study when Kefford *et al*. (2004) exposed *C. nilotica* to $Na_2SO_4$, in acute experiments $Na_2SO_4$ was more toxic than NaCl. Goetsch & Palmer (1997) investigated the salinity tolerance of macroinvertebrates in the Sabie River, and they discovered that the mayfly nymphs (*Tricorythus tinctus)* were more sensitive to $Na_2SO_4$ than to NaCl. The same trend is revealed in the UCEWQ database (Table 4.1). Table 4.5 shows the NOEC and LOEC values with the endpoints which were used to establish these values being the decrease in length, width, and area of the egg, yolk and optic rudiment of the developing embryo.

Table 4.5 The LOEC and NOEC values of the effects of $Na_2SO_4$, NaCl and $CdCl_2$ on the embryonic development of *C. nilotica*

| **Toxicant** | **time (hrs)** | **Effect** | **Feature** | **Conc (mg/l)** |
|---|---|---|---|---|
| $Na_2$ $SO_4$ | 144 | NOEC | | 1000 |
| | | LOEC | yolk, optic rudiment | 2000 |
| NaCl | 216 | NOEC | | 2000 |
| | | LOEC | yolk, egg, antennae antennule and optic rudiment | 3000 |
| $CdCl_2$ | 24 | NOEC | | <0.31 |
| | | LOEC | yolk | 0.31 |

When Kefford *et al.* (2004) investigated the salinity tolerance of eggs and hatchlings of selected aquatic macroinvertebrates in south-east Australia and South Africa they came to the conclusion that the mean tolerance of the most sensitive younger life stage was barely half that of older life stages. Lee *et al*. (2000) suggested that this may be due to the fact that there is intense cellular activity and organ development over a relatively short period during embryonic development: it has been suggested that during cell division the more open chromosome is more susceptible to toxicant entrance. Thus, embryos exposed to toxicants for short periods may be more sensitive than longer exposure in later life stages.

Slaughter (2005) found the NOEC for $Na_2SO_4$ to be 1900 mg/L and the LOEC to be 2530mg/L for growth and reproduction of *C. nilotica* over a period of 80 days. For NaCl the NOEC was 1900mg/L and the LOEC was 2200mg/L for growth, and for reproduction the NOEC was 1900mg/L and LOEC was 2700mg/L. Compared to the LOEC and NOEC values found in this study, the NOEC and LOEC values for NaCl found by Slaughter (2005) were lower. For $Na_2SO_4$ the NOEC and LOEC values found in this study were lower than the values found by Slaughter (2005).

The $CdCl_2$ concentrations were the most lethal to the embryos, as the mortality rate was higher even within the lowest concentrations. In other studies it has been determined that when older aquatic invertebrates were exposed to cadmium there was a decrease in growth and fecundity (Coetzee, 1989; de Nicola Guidici *et al*. 1987; Kogan *et al.* 2000; Lee *et al*., 1996; Muyssen and Janssen, 2004; Rodríguez and Medesani, 1994; Witeska *et al*., 1995). A full lifecycle test would allow the observation of the reproduction of the embryos that did hatch when they reach the mature stage. In a study undertaken by Muyssen and Janssen (2004), *Daphnia magna* were exposed to cadmium in acute and chronic tests. They found that *D. magna* in acute test became acclimated to the toxicant whereas in chronic tests there was a decrease in survival and reproduction, which shows the importance of a chronic toxicity method.

Further tests need to be done for NaCl, $Na_2SO_4$ and $CdCl_2$ and for other toxicants before embryonic development can be considered for regulatory testing. However, the reduction in length, width and area of the identified measured features show potential as an experimental endpoint with which to determine an effect on developing embryos of *Caridina nilotica*.

# CHAPTER 5

## Discussion and Conclusions

Standard test protocols using laboratory-reared organisms which are indigenous to South Africa are limited (DWAF, 2000) and protocols for culturing indigenous test organisms in South African facility are in the process of being developed and refined (Haigh and Davies-Coleman, 1997, 1999; Muller *et al.,* in press). The standard toxicity tests can be used to generate toxicity data that can be used to formulate water quality guidelines that are directly applicable to South African aquatic ecosystems (DWAF, 2000). Indigenous species such as *C. nilotica* are currently being considered as standard toxicity test organisms.

The sub-lethal chronic effects of long-term exposure of lower concentrations of toxicants on invertebrates and other aquatic organisms are not well documented (Hart *et al.*, 1990, Kefford, 1998). It is therefore necessary to have chronic test data because it can provide a more environmentally realistic measure of chemical toxicity than acute toxicity tests, as chronic test data are regarded as more relevant for the setting of environmental water quality guidelines. However, acute response data are often used to extrapolate chronic responses (Slaughter, 2005), hence the study presented here could make a valuable contribution to the development of a chronic toxicity database, particularly for indigenous species.

Chronic toxicity data for indigenous aquatic organisms can play an important role in updating the present guidelines, to be more representative of South African aquatic biota, since the South African Water Quality Guidelines (DWAF, 1996) are based mainly on international toxicity data. The process of developing and refining environmentally realistic water quality guidelines in South Africa for all water variables is a complex one, but should remain under review as more data are gathered using indigenous organisms for toxicity tests and as methods for applying these data in water resource management are refined (Browne, 2005). In this study a chronic toxicity test method for an indigenous species was

developed and contributed sub-lethal chronic toxicity data to the developing database.

*Caridina nilotica* is readily reared under laboratory conditions, and breeds throughout the year. The biology and life history of the species has also been researched (Hart, 1980; Hart 1981; Hart, 1982, Hussein and Obuid-Allah, 1992a, Hussein and Obuid-Allah, 1992b), but limited information is available. Food suitable for culturing this species in the laboratory is also readily available and inexpensive (Chapter 2). Early life stages are presumed to be sensitive to various concentrations of chemicals (Rand, 1995; Kefford *et al.*, 2004). The purpose of this study was therefore to develop a chronic toxicity test method to allow toxicity tests of embryonic development of *C. nilotica.*

**5.1 Experimental brood chambers**

The experimental brood chambers allowed incubation and subsequent survival of the embryos until they hatched. Removing eggs from recently gravid *C. nilotica* females and placing them inside the newly developed experimental brood chambers did not appear to result in any negative effects on the developing embryos. The developmental rate of the embryo in the experimental brood chamber was no different to when eggs were carried by the female from spawning to hatching. Before the actual embryonic development of *C. nilotica* was monitored, gravid females were put in separate tanks to determine the duration of the embryonic development at 24℃ (Chapter 3). To observe if development is the same between eggs that were removed from the females and those that remained with the female, eggs could be removed from gravid females and embryonic development monitored, and then compared to the development of eggs held by female.

Due to the fact that varying water quality parameters can introduce variability in the test endpoints (Celada *et* al., 1991; Cooney, 1995), selected water quality parameters such as temperature, electrical conductivity, dissolved oxygen and

pH were measured for the duration of each experiment. When the water quality parameters were compared to the values obtained in the culture tanks where the *C. nilotica* are bred and reared, it showed that variability of these measured factors was kept to a minimum (Chapter 2, Figs. 2.3-2.6). The fact that the rate of embryonic development in the brood chamber was the same as in culture tanks and that the water quality variables within the brood chambers remained within the normal range of the culture tanks suggested that this would be an appropriate test method for embryonic development.

### 5.2 Embryonic development of *Caridina nilotica*

During embryonic development of *C. nilotica,* different developmental ages could be observed readily due to the transparency of the egg and the large size of the embryo. There was an increase in size of eggs from spawning until hatching; in other embryonic development studies this was associated with, among other factors, an increase of water content in eggs (Kobayashi and Matsuura, 1995; Lavaris *et al.*, 2002). As the embryo develops, the egg membrane alters its permeability during development, probably allowing water uptake (Kobayashi and Matsuura, 1995; Lavaris *et al.*, 2002). This uptake of water may result in uptake of toxins in the water, with subsequent consequences to the developing embryo. This could be a good reason that the embryo test might be an appropriate and possibly sensitive toxicity test method.

The results obtained for the embryonic development of *C. nilotica* in chapter 3 provided a valuable contribution to the study because the identified development ages were easily recognised. At the same time, the easy measurement of the various features of the developing embryos also provided a useful contribution to the study. Thus, major morphological events during the development of embryos could be followed, which was useful in determining the effects of the selected toxicants on the developing embryo. Variability was kept to a minimum as all the eggs were spawned within 12 hours of one another (Chapter 2).

Nair (1949) studied the embryonic development of *Caridina laevis*, he removed a few eggs daily from the gravid females, placed them in a watch glass with small amount of water and monitored the development by removing as much of the yolk as possible. The identified embryonic development ages of *C. laevis* were the same as those of *C. nilotica* identified in this study. Although the development of *C. laevis* also took 17 days (Nair, 1949), the temperature of the tanks where females were bred was not mentioned, and therefore more comprehensive comparisons to *C. nilotica* were not possible.

Chinnayya (1974) monitored the embryonic development of *Caridina weberi* with temperatures ranging from 24-26℃. The larvae hatched on the 15$^{th}$ day, which was 2 days earlier than reported for *C. nilotica*. However, in this study, the water temperatures were not allowed to reach 26℃ and this may be the cause of reduced duration of embryonic development of *C. weberi*. Developmental features of *C. weberi* were the same as those identified for *C. nilotica* (Chapter 3). However Chinnayya (1974) mentioned when the features were fully developed and not the first appearance of the features, nor was there mention of how it was established whether the features were fully developed. It might be better to identify the initial appearance of the feature in order to know as soon as possible when a response occurs to the developing embryo, in addition to establishing what the effects are on the developmental feature.

The embryonic development of *Palaemonetes argentinus* studied by Nazari *et al.* (2000) at 23℃ showed that embryonic development took 10 days. The main feature mentioned in the study by Nazari *et al.* (2000) was the appearance of the eye; each stage was defined by the size and shape of the eye. Lavarias *et al.* (2002) did a morphometric study of the embryonic development of *Macrobrachium borellii* at 24℃ and the embryos hatched after 39 days. Area, perimeter, maximum and minimum diameters and shape were measured in yolk sac, egg coat and eye respectively. The measurements were useful in this research but we also measured other features which were not identified in Lavarias *et al.* (2002).

While descriptions of developmental period and features for other species provided useful guidelines, the details of these needed to be established for *C. nilotica* which is an indigenous species. This study determined whether the embryonic development of *C. nilotica* could be considered a suitable early life stage toxicity test method.

### 5.3 Using embryonic development of *C. nilotica* as a chronic toxicity test method

The eggs are relatively easy to remove from the female and to handle. They are readily transferable from the females to the experimental brood chambers using a soft paint brush and a pipette, and can be removed easily from the experimental brood chambers to monitor embryo development.

The results of this study demonstrated that the easily identified developmental ages and morphological features of *C. nilotica* could be used in toxicity tests. Using a replicated experimental design and an ANOVA statistical analysis, statistically significant effects could be found, and ascribed to exposure to the toxicants. *C. nilotica* showed more tolerance to NaCl than to the other two chemicals tested. Similar studies by Goetsch & Palmer (1997) investigated the salinity tolerance of macroinvertebrates in the Sabie River to NaCl and $Na_2SO_4$. Mayfly nymphs (*Tricorythus tinctus*) were discovered to be more sensitive to $Na_2SO_4$ than to NaCl. Data in the UCEWQ database (Table 4.1) also shows that NaCl is less toxic than $Na_2SO_4$.

The LOEC observed for NaCl was 3000mg/L at 216 hours and the NOEC was 2000mg/L, the morphological features which allowed the establishment of these values were the measured sizes of the egg, yolk, antenna, antennule and optic rudiment. The total percentage of the embryos that hatched after 408 hours was 33% and they hatched from concentration 1000mg/L to 3000mg/L. The $Na_2SO_4$ was shown to be more toxic than the NaCl. The LOEC of $Na_2SO_4$ was 2000mg/L at 144 hours, and the estimated NOEC was 1000mg/L, with the sizes of the yolk

and the optic rudiment being the features which allowed the identification of the LOEC/NOEC values. The total percentage of the embryos that hatched after 408 hours was 18% and all the embryos were from the concentration of 1000mg/L. The results presented for $CdCl_2$ indicate higher toxicity than the other two toxicants. The NOEC for $CdCl_2$ was found to be less than 0.31mg/L and the LOEC was 0.31mg/L at 24 hours, with the yolk being the morphological feature which allowed identification of the NOEC/LOEC values. A total of 2.6% of the eggs hatched after 408 hours and they were from 0.31mg/L concentration. In order to establish a NOEC for $CdCl_2$, further experimental work must be undertaken.

Comparing the NOEC values of this study and the NOECs in the UCEWQ database and Slaughter (2005) for $Na_2SO_4$, it is possible to conclude that the early life stages of *C. nilotica* are more sensitive to chemicals than the older life stages for $Na_2SO_4$. The NOEC for $Na_2SO_4$ in my study was 1000mg/L and in the UCEWQ database and in Slaughter (2005) it was 1900mg/L. However, for NaCl I found the NOEC for my study to be 2000mg/L and Slaughter (2005) found the NOEC value of 1900mg/L. Choosing concentrations between 1000mg/L and 2000mg/L might give us an NOEC less than 1900mg/L, this still needs to be considered. The LC50s of $CdCl_2$ for older life stages of *C. nilotica* ranged from 0.05 – 0.09 mg/L from the UCEWQ database which, shows that lower concentrations need to be considered. However, based on the membrane of the *C. nilotica* eggs and the unknown toxicity of the $CdCl_2$ on the eggs a wide range of concentrations to test were chosen in this range finding study.

Data for the effects of NaCl and $Na_2SO_4$ on embryonic development of other freshwater shrimps could not be found. The combined effects of temperature and Cd on developmental parameters and biomarker responses in zebrafish (*Danio rerio*) embryos showed the $LC_{50}$ at 26℃ being 30.1mg/L (Hallare *et al*., 2005). This value is very high, suggesting that the embryos of *C. nilotica* are more sensitive than the embryos of the zebrafish. Botton (2000) studied the toxicity of cadmium and mercury to horseshoe crab (*Limulus polyphemus*) embryos and

larvae, and for embryos the $LC_{50}$ value was 0.5mg/L at 48 hours and 0.2mg/L at 72 hours; no NOEC values are presented. However the $LC_{50}$ values at 72 hours are lower than the LOEC values found in my study. Since horseshoe crabs are a marine species, it is not clear whether differences can be ascribed to these different environments.

### 5.4 Conclusion

Salinity tolerance information for indigenous species is still important for setting salinity water quality guidelines in South Africa. The NOECs found in this study were compared with NOEC values of different test methods, to give an indication of the sensitivity of the test method developed in this study. Based on results from this study, *Caridina nilotica* is an excellent indigenous organism to use for chronic toxicity tests where growth of embryos as a biological response is measured although further testing of other chemicals is required to verify this. Whether teratogenic effects such as lesions, head malformation and spinal curvatures found in other studies (Rodríguez and Medesani, 1994, Witeska, 1995) can be found in *C. nilotica* still needs to be determined.

On the whole, the results suggest that the embryonic development of *C. nilotica* can be a valuable indicator of contamination in freshwaters by aging the dead eggs and comparing their morphometry to the experimental values. Morphometric measurements of the developmental features could be an indication of an effect caused by chemicals. However, it would be necessary to extend this study to other pollutants to achieve more generalized conclusions useful for water quality monitoring. Embryonic development of *C. nilotica* could possibly be used in future studies in which the toxicity of complex effluents to the developing embryo is assessed. A preliminary experiment was undertaken during this study, exposing the eggs of *C. nilotica* to whole effluent collected from a textile industry. Results from this preliminary assessment suggest that the method developed in this study may be useful in the toxicity assessment of complex effluents.

The present results represent a stimulus for further studies of the effects of chemicals on developing embryos, including the establishment of teratogenic effects of aquatic pollutants on *C. nilotica* embryos. The results presented in this study will contribute to the development of chronic toxicity data for *C. nilotica* in the UCEWQ database, and contributes to national and international data available for the development of environmentally realistic water quality guidelines for aquatic ecosystems.

## CHAPTER 6

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# APPENDICES

## APPENDIX A

### Intrafemale variability

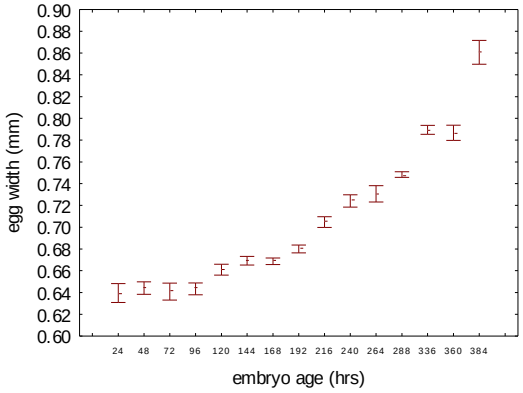


**Fig. A** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the egg width (mm) of *C. nilotica* over time

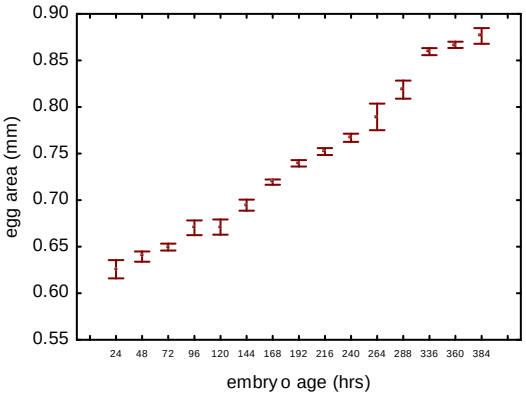


**Fig. B** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the egg area (mm) of *C. nilotica* over time

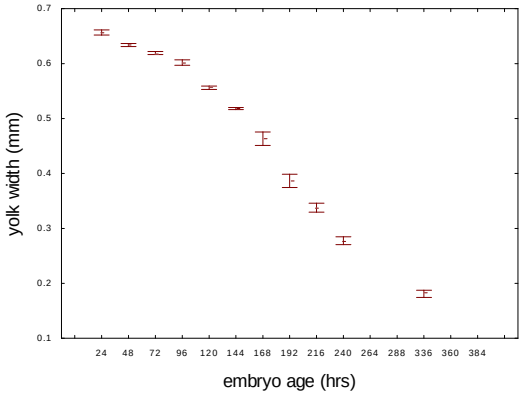


**Fig. C** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the yolk width (mm) of *C. nilotica* over time

**Interfemale variability**

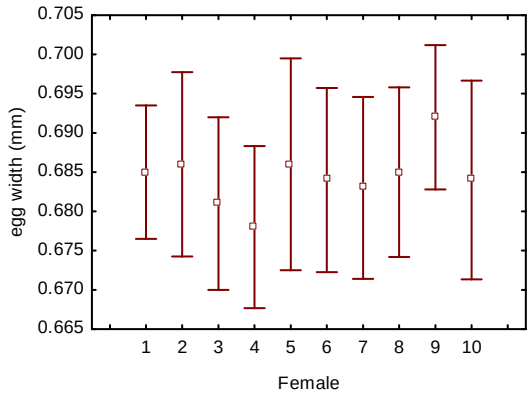


**Fig. A** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the egg width (mm) of embryos from different females of *C. nilotica* at 72 hours

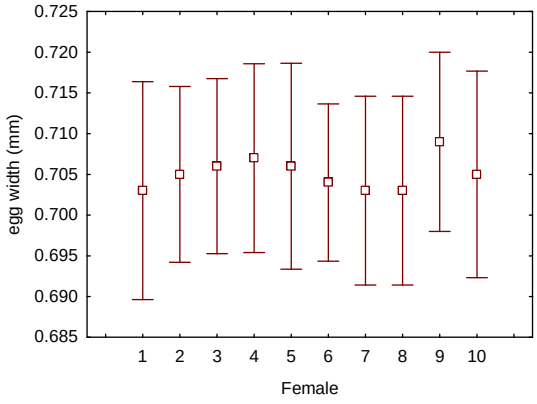


**Fig. B** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the egg width (mm) of embryos from different females of *C. nilotica* at 216 hours

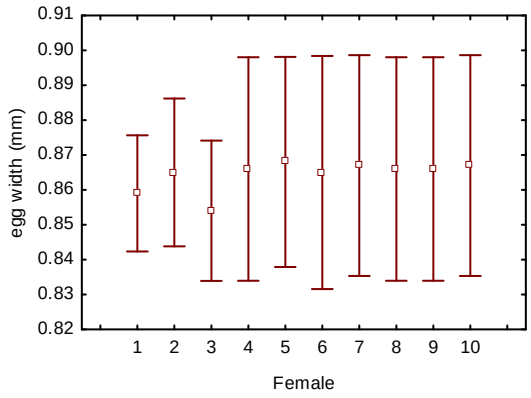


**Fig. C** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the egg width (mm) of embryos from different females of *C. nilotica* at 384 hours

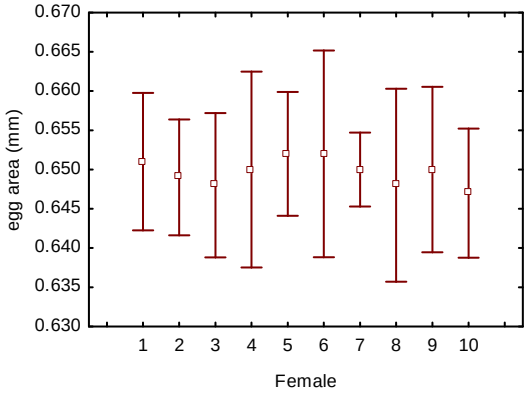


**Fig. D** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the egg area (mm) of embryos from different females of *C. nilotica* at 72 hours

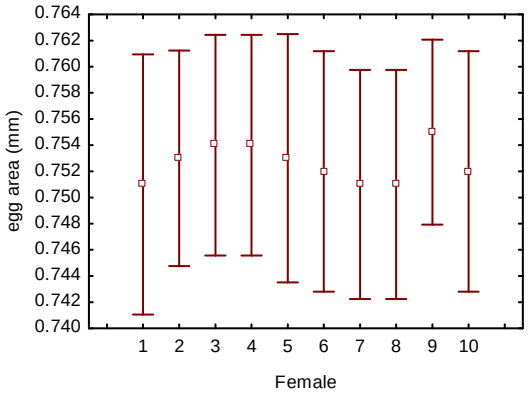


**Fig. E** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the egg area (mm) of embryos from different females of *C. nilotica* at 216 hours

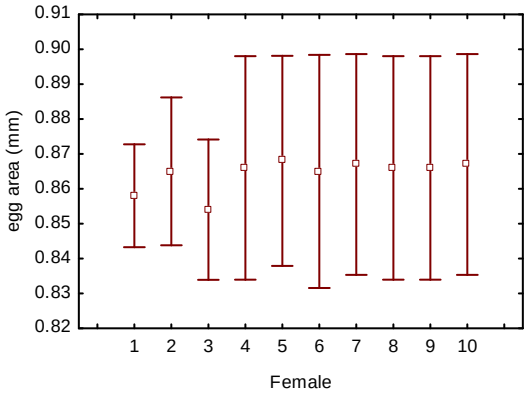


**Fig. F** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the egg area (mm) of embryos from different females of *C. nilotica* at 384 hours

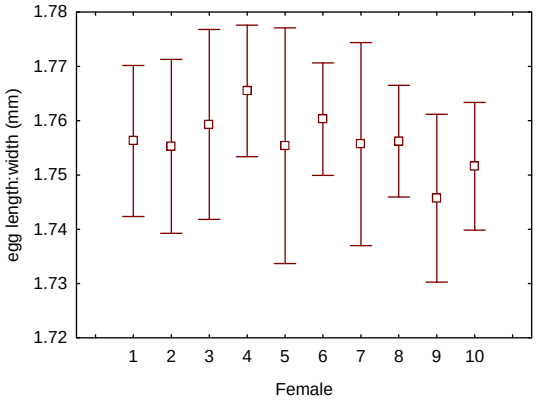


**Fig. G** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the egg length:width ratio (mm) of embryos from different females of *C. nilotica* at 72 hours

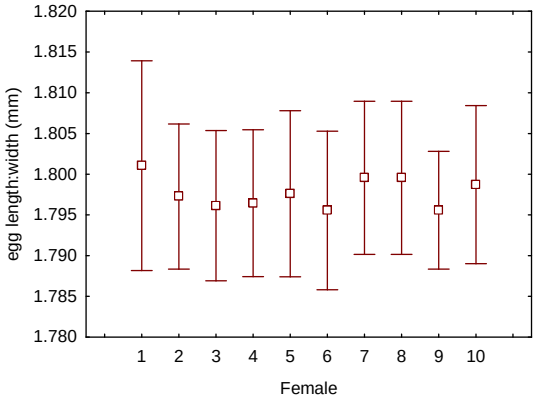


**Fig. H** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the egg length:width ratio (mm) of embryos from different females of *C. nilotica* at 216 hours

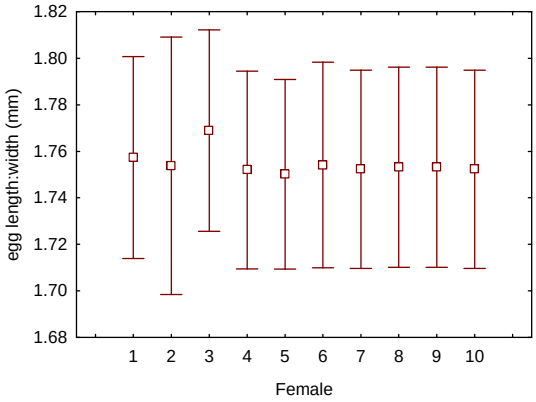


**Fig. I** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the egg length:width ratio (mm) of embryos from different females of *C. nilotica* at 384 hours

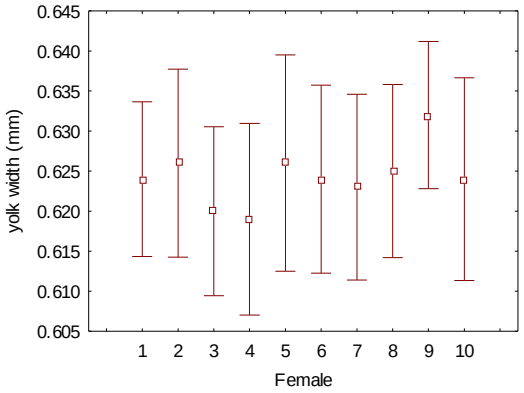


**Fig. J** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the yolk width (mm) of embryos from different females of *C. nilotica* at 72 hours

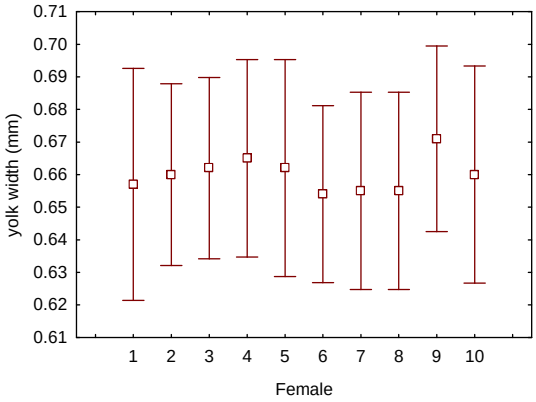


**Fig. K** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the yolk width (mm) of embryos from different females of *C. nilotica* at 216 hours

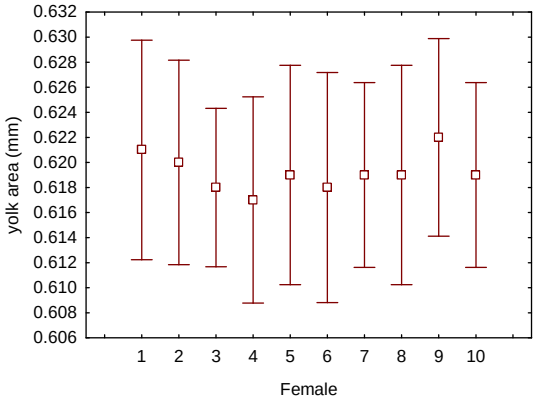


**Fig. L** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the yolk area (mm) of embryos from different females of *C. nilotica* at 72 hours

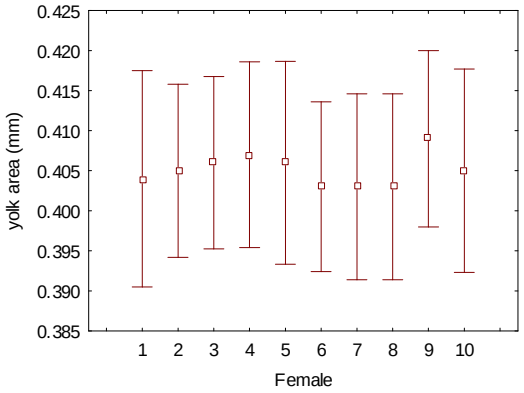


**Fig. M** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the yolk area (mm) of embryos from different females of *C. nilotica* at 216 hours

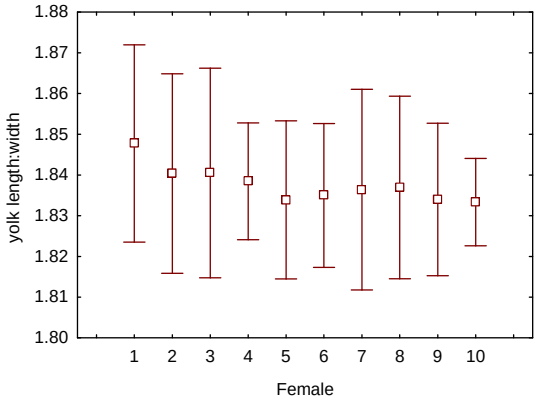


**Fig. N** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the yolk length:width ratio of embryos from different females of *C. nilotica* at 72 hours

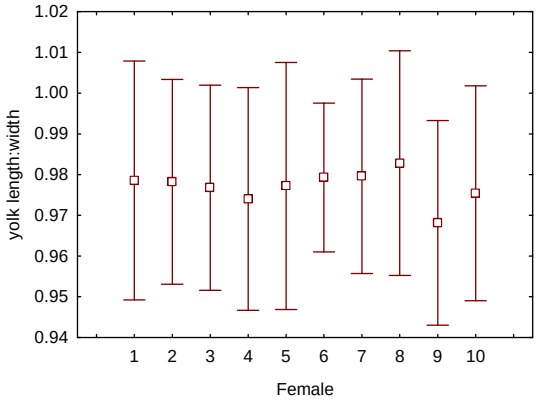


**Fig. O** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the yolk length:width ratio of embryos from different females of *C. nilotica* at 216 hours

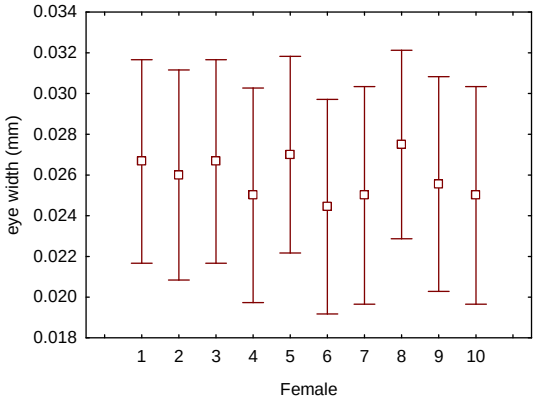


**Fig. P** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the of the eye width of embryos from different females of *C. nilotica* at 216 hours

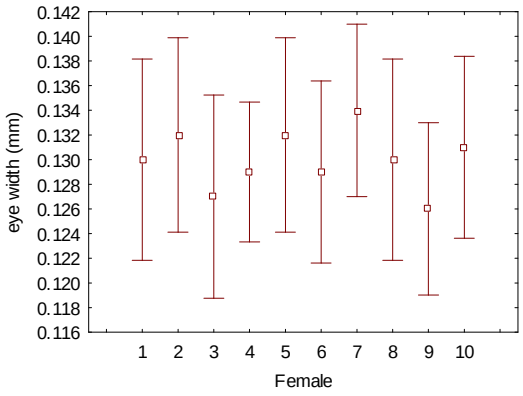


**Fig. Q** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the of the eye width of embryos from different females of *C. nilotica* at 384 hours

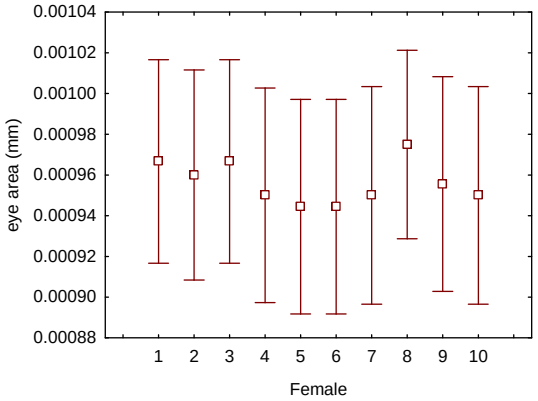


**Fig. R** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the of the eye area of embryos from different females of *C. nilotica* at 216 hours

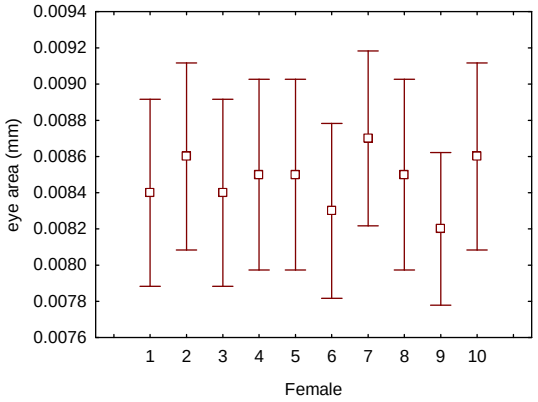


**Fig. S** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the of the eye area of embryos from different females of *C. nilotica* at 384 hours

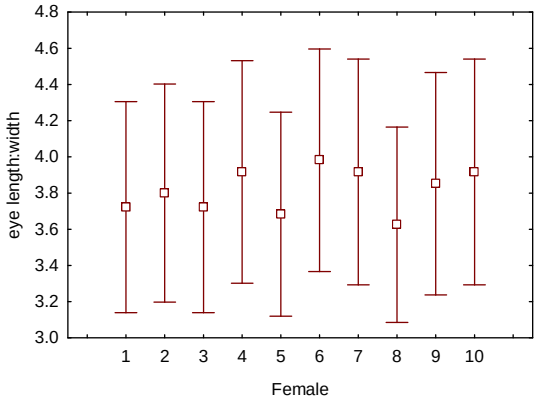


**Fig. T** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the of the eye length:width ratio of embryos from different females of *C. nilotica* at 216 hours

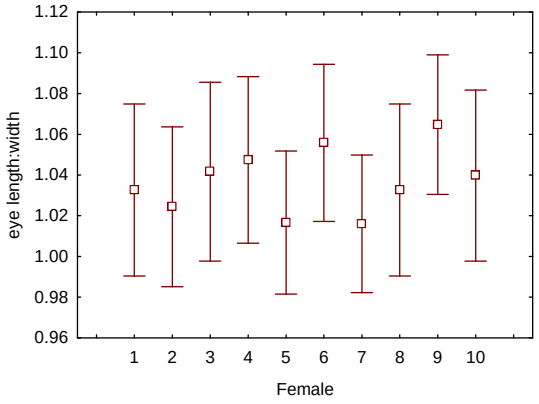


**Fig. U** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the of the eye length:width ratio of embryos from different females of *C. nilotica* at 384 hours

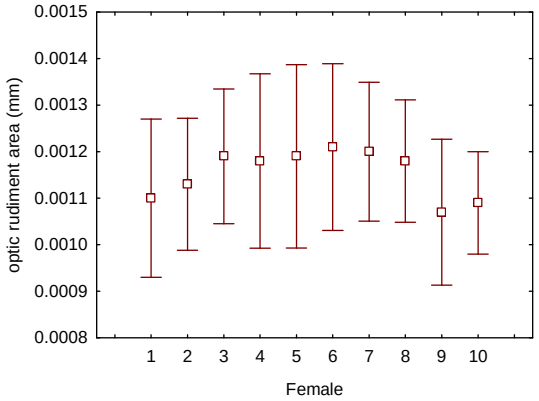


**Fig. V** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the of the optic rudiment area of embryos from different females of *C. nilotica* at 72 hours

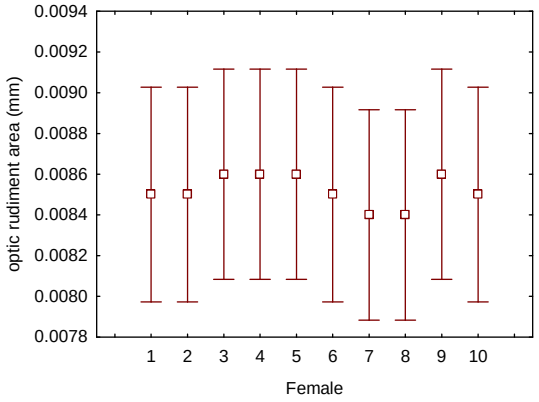


**Fig. W** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the of the optic rudiment area of embryos from different females of *C. nilotica* at 216 hours

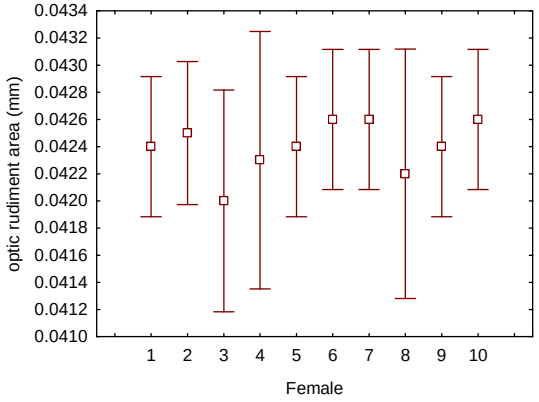


**Fig. X** Whisker plots showing the means (□) and standard deviations (indicated by whiskers) of the of the optic rudiment area of embryos from different females of *C. nilotica* at 384 hours

## APPENDIX B

### $Na_2SO_4$, NaCl and $CdCl_2$

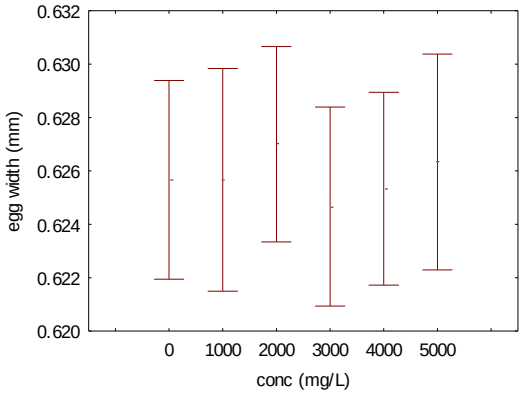


**Fig. A** Mean egg width (mm) for the embryos of *C. nilotica* at 72 hours, shows there is no difference between the concentrations of $Na_2SO_4$ and the control. Lines show the standard deviations of the egg length in the different concentrations.

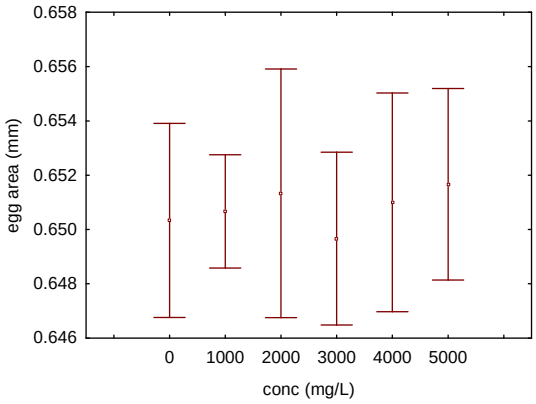


**Fig. B** Mean egg area (mm) for the embryos of *C. nilotica* at 72 hours, shows there is no difference between the concentrations of $Na_2SO_4$ and the control. Lines show the standard deviations of the egg length in the different concentrations.

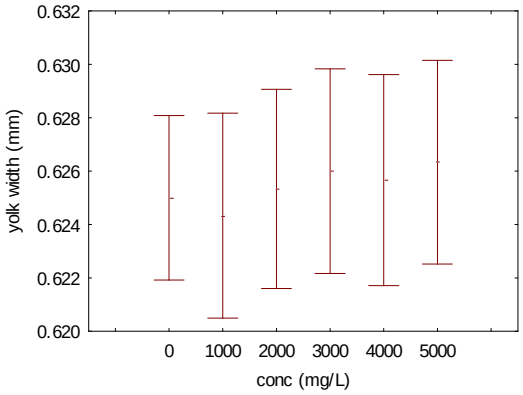


**Fig. C** Mean yolk width (mm) for the embryos of *C. nilotica* at 72 hours, shows there is no difference between the concentrations of $Na_2SO_4$ and the control. Lines show the standard deviations of the egg length in the different concentrations.

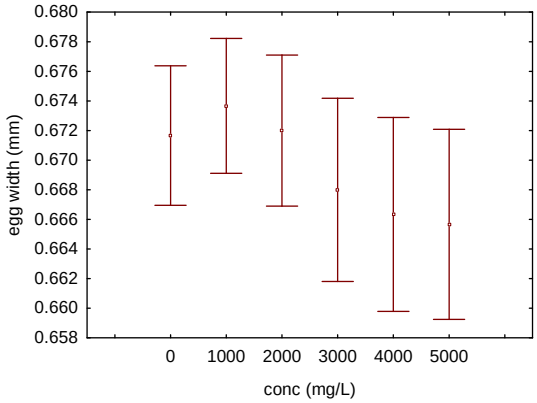


**Fig. D** Mean egg width (mm) for the embryos of *C. nilotica* at 144 hours, shows a decrease in egg width at 3000mg/L to 6000mg/L of $Na_2SO_4$. Lines show the standard deviations of the egg width in the different concentrations

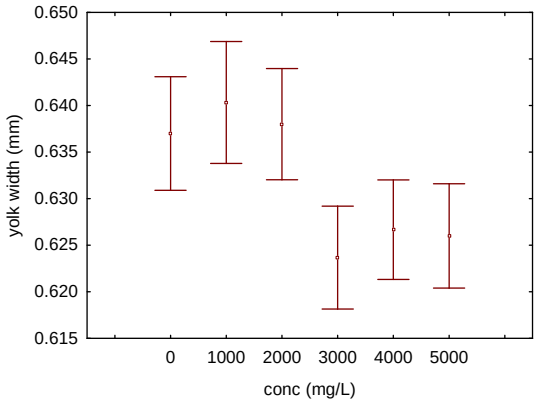


**Fig. E** Mean yolk width (mm) for the embryos of *C. nilotica* at 144 hours, shows a decrease in egg width at 3000mg/L to 6000mg/L of $Na_2SO_4$. Lines show the standard deviations of the yolk width in the different concentrations

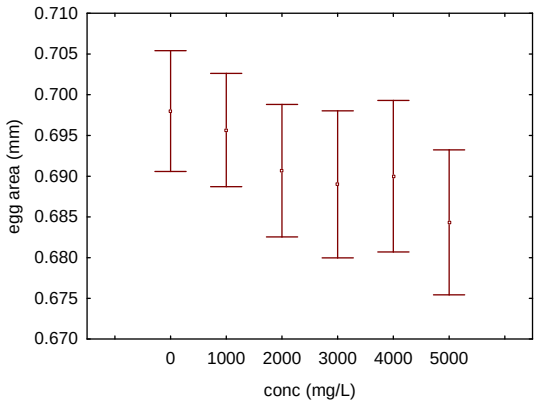


**Fig. F** Mean egg area (mm) for the embryos of *C. nilotica* at 144 hours, shows a decrease in egg width at 3000mg/L to 6000mg/L of $Na_2SO_4$. Lines show the standard deviations of the egg area in the different concentrations

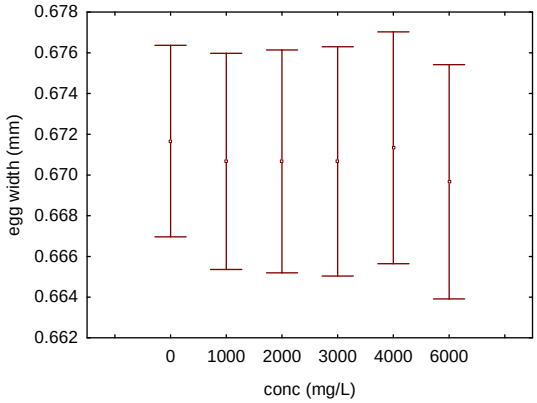


**Fig. G** Mean egg width (mm) of embryos of *C. nilotica* at 144 hours, shows there is no difference in egg width between concentrations of NaCl and control. Lines show the standard deviations of the egg width in the different concentrations

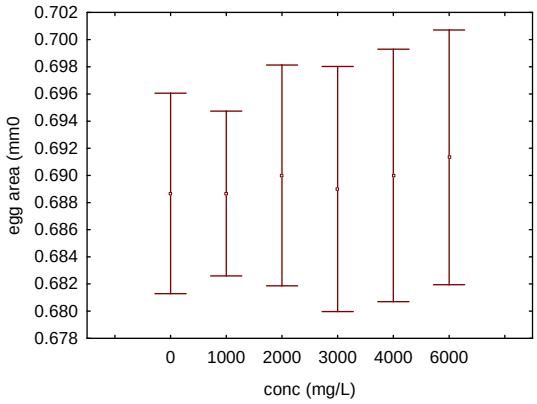


**Fig. H** Mean egg area (mm) of embryos of *C. nilotica* at 144 hours, shows there is no difference in egg area between concentrations of NaCl and control. Lines show the standard deviations of the egg area in the different concentrations

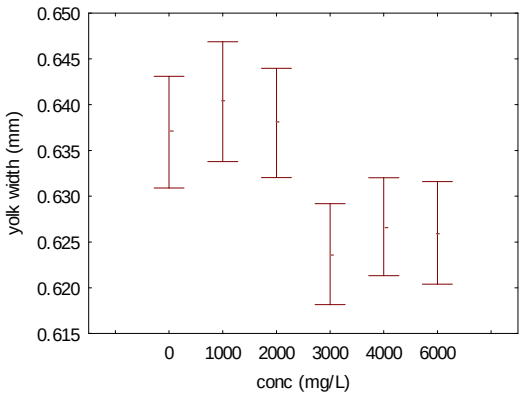


**Fig. I** Mean yolk width (mm) of embryos of *C. nilotica* at 216 hours, shows a decrease in yolk width at 3000mg/L to 6000mg/L of NaCl. Lines show the standard deviations of the yolk width in the different concentrations

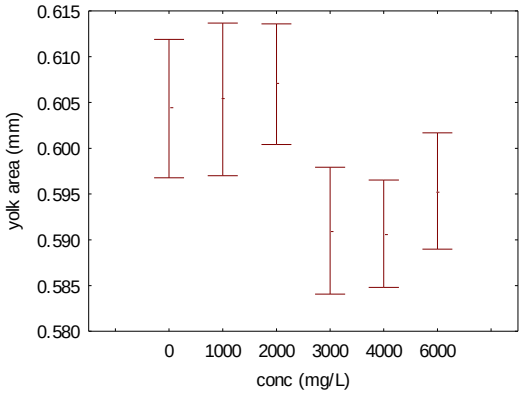


**Fig. J** Mean yolk area (mm) of embryos of *C. nilotica* at 216 hours, shows a decrease in yolk area at 3000mg/L to 6000mg/L of NaCl. Lines show the standard deviations of the yolk area in the different concentrations

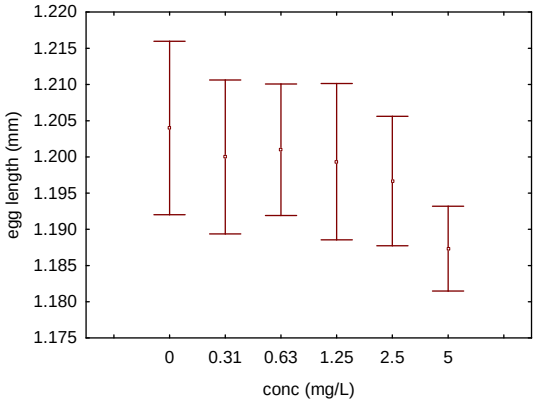


**Fig. K** Mean egg length (mm) of embryos of *C. nilotica* at 24 hours, shows a decrease in egg length at 5mg/L of $CdCl_2$. Lines show the standard deviations of the egg length in the different concentrations

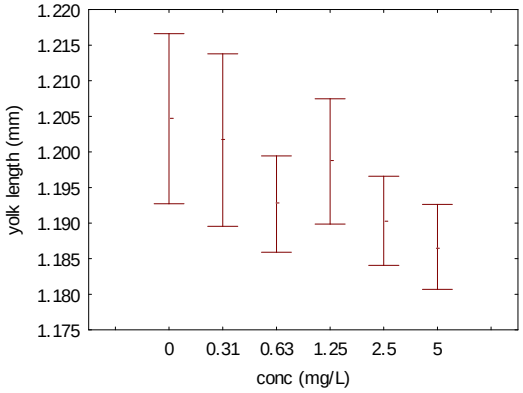


**Fig. L** Mean yolk length (mm) of embryos of *C. nilotica* at 24 hours, shows a decrease in yolk length at 0.63mg/L to 5mg/L of $CdCl_2$. Lines show the standard deviations of the yolk length in the different concentrations

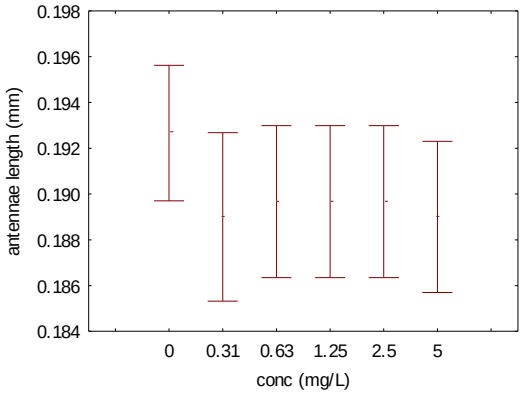


**Fig. M** Mean antennae length (mm) of embryos of *C. nilotica* at 72 hours, shows a decrease in antennae length in all concentrations of $CdCl_2$. Lines show the standard deviations of the antennae length in the different concentrations

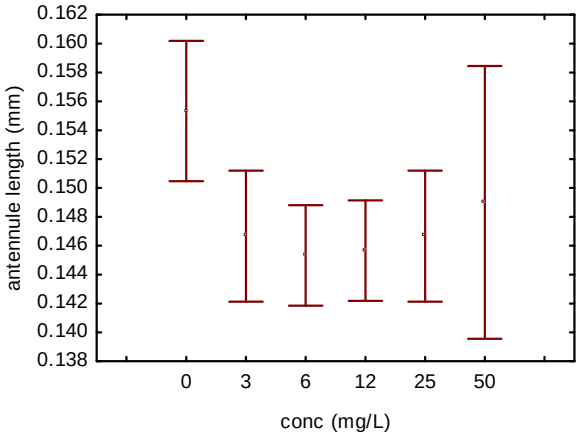


**Fig. N** Mean antennule length (mm) of embryos of *C. nilotica* at 144 hours, shows a decrease in antennule length in all concentrations of $CdCl_2$. Lines show the standard deviations of the antennule length in the different concentrations