Differences between saline-injected and CS extract-injected specimens were evaluated using Student's t-test.

Results. On injection of CS extract the serum calcium level decreases at 0.5 h in both the species (table). This response intensities progressively up to 2 h in *Rana cyanophlyctis* ($p < 0.001$) and up to 4 h in *Bufo andersonii* ($p < 0.001$). At 6 h higher calcemic values have been recorded (*R. cyanophlyctis*, $p < 0.025$; *B. andersonii*, $p < 0.005$). By 8 h serum calcium returns to normal levels.

Discussion. The hypocalcemic effect of CS extract has been reported earlier in eels[3] and in *Fundulus heteroclitus*[1]. The present study clearly indicates that CS extract induces hypocalcemia in anurans as well. The hypocalcemic effect of CS extract in non-piscine vertebrates receives further support from the recent studies in rats[9] and parrots[10]. However, reports of the effect of CS extract administration on serum calcium of rats are contradictory. Milet et al.[11] have reported hypercalcemia whereas Pang (cited by Leung and Fenwick[9]) and Copp (cited by Leung and Fenwick[9]) have failed to obtain any response.

The hypercalcemia observed in the present study at 6 h may be due to the activity of the parathyroid glands in response to the CS extract-induced hypocalcemia.

1 Pang, P.K.T., Pang, R.K., and Sawyer, W.H., Endocrinology *94* (1974) 548.
2 Ma, S.W.Y., and Copp, D.H., in: 'Comparative endocrinology', p.283. Eds P.J. Gaillard and H.H. Boer. Elsevier/North-Holland Biomedical Press, Amsterdam 1978.
3 Fontaine, M., C. r. Acad. Sci. Paris D *259* (1964) 875.
4 Chan, D.K.O., Gen. comp. Endocr., suppl. *3* (1972) 411.
5 Fenwick, J.C., Gen. comp. Endocr. *23* (1974) 127.
6 Fenwick, J.C., and Forster, M.E., Gen. comp. Endocr. *19* (1972) 184.
7 Pang, P.K.T., Pang, R.K., and Sawyer, W.H., Endocrinology *93* (1973) 705.
8 Trinder, P., Analyst *85* (1960) 889.
9 Leung, E., and Fenwick, J.C., Can. J. Zool. *56* (1978) 2333.
10 Srivastav, A.K., and Swarup, K., Experientia *38* (1982) 869.
11 Milet, C., Lopez, E., Chartier, M.M., Martelly, E., Lallier, F., and Vidal, B., in: Molecular endocrinology, p.341. Eds I. MacIntyre and M. Szelke. Elsevier/North-Holland Biomedical Press, Amsterdam 1979.

Embryonic diapause in annual fishes: evaporative water loss and survival

J.R. Matias

Orentreich Foundation for the Advancement of Science, 910 Fifth Avenue, New York (New York 10021, USA), 2 April 1982

Summary. The effects of partial desiccation on the survival of diapause I, diapause II, and pre-hatching embryos of the annual fish *N. guentheri* were investigated. Embryos at diapause II were found to be the most resistant stage. Prolonged exposure of diapause II embryos to 92 and 95% relative humidities retarded the termination of diapause II.

The East African annual fish, *Nothobranchius guentheri* (Pisces; Cyprinodontidae), inhabits isolated bodies of fresh water that are normally subject to periodic drought[1,2]. The evaporative water loss during the dry season results in the death of adult and juvenile fishes. The population escapes extinction in the form of thickly chorionated embryos encased in the bottom substrate which enter a state of developmental arrest or diapause at specific stages of their normal ontogeny[2,3].

A variety of factors, such as temperature[4], photoperiod[5,6] and oxygen tension[3,7], have been demonstrated to control the onset and the duration of diapause. But knowledge of the role of partial desiccation on the survival and development of diapausing embryos have been lacking. The present work was therefore undertaken to determine the influence of partial desiccation on diapause I, diapause II, and pre-hatching embryos by exposure to a series of controlled relative humidities.

Materials and methods. The source of fish, husbandry conditions, and method of embryo collection have already been described in detail for *N. guentheri*[4,8]. Breeding pairs were maintained at a photoperiod of 14L:10D at 20±1 °C. Fertilized embryos were collected and incubated at 20 °C in 125 ml Ehrlenmeyer flasks containing 100 ml of aquarium water. This temperature has been shown to induce longer sojourn in diapause I and entry into diapause II.

A series of controlled relative humidities (RH) was achieved by using graded solutions of potassium hydroxide as described by Solomon[9]. Groups of 20 to 25 embryos were selected, blotted until no water was visible, and placed on a Whatman filter paper cut to fit inside a 60-mm petri dish. The embryos were immediately transferred to a 160-mm glass desiccator containing 100 ml of the appropriate potassium hydroxide solution. After the replacement of the lid, faster equilibration of the humidity level was achieved by the use of a magnetic stirring bar for 5 min to agitate the solution at the bottom. The humidified chamber was then placed in an incubator at a constant temperature of 25 °C. Aquarium water was added to the petri dishes and the number of surviving embryos were counted 24 h later.

Embryos were exposed to nonlethal RH for extended periods to determine the effect of partial desiccation on the duration of diapause. The embryos were removed from the humidity chamber at selected time points and water was added immediately to the petri dishes to prevent total desiccation. The nomenclature established by Wourms[10] to describe the stages of development of the annual fish *Austrofundulus myersi* Dahl was used. After the determination of the developmental stages under a dissecting microscope the embryos were returned to the humidity chamber by the procedure described above. Controls represent those embryos placed on filter paper saturated with water inside a tightly sealed petri dish and incubated at 25 °C.

The frequency distribution of the stages of development among diapause II embryos maintained for 10 days in various relative humidities (RH) at 25 °C

		Stages of development			
	(n)	Diapause II	33–34	36–37	39–40
Control	54	18.5	20.4	46.3	14.8
97% RH	23	43.5	34.8	21.7	–
92% RH	31	45.2	38.7	16.1	–

Results. Embryos at diapause I, diapause II and pre-hatching embryos were exposed to varying levels of humidity for 3 h and then hydrated by the addition of aquarium water. The data strongly indicated that diapause II embryos were the most resistant to decreasing RH (fig. 1) while embryos at diapause I were extremely sensitive to the treatment. Contraction and flattening of the embryos occur when exposed to RH lower than 90%. However, upon addition of water the embryos resume their normal morphology within 10 min. Those that survive the brief desiccation continue their normal development when hydrated and hatch without any visually detectable abnormalities. No mortality was observed among the control embryos.

The degree of resistance demonstrated by diapause II embryos was further examined by longer exposure to various RH. At 92% no mortality was observed even after 120 h of continuous exposure. However, at 80 and 70% RH a progressive decline in survival is quite evident (fig. 2).

To determine whether exposure to partial desiccation influences the duration of diapause, embryos that are undergoing diapause II at 20 °C were exposed for extended periods to 92 and 97% RH at 25 °C. The transfer to a higher temperature normally breaks diapause II and stimulates the resumption of organogenesis. The data in figure 3 demonstrates that the commencement of organogenesis was retarded by the exposure to partial desiccation. The curve generated for the embryos exposed to 92 and 97% RH were virtually identical. The time at which 50% of the control and experimental populations exited from diapause II were 7.0 and 9.5 days, respectively.

The frequency distribution of the stages of development after 10 days of exposure to various humidities are presented in the table. At this time the vast majority of the control embryos had already exited from diapause II and had reached more advanced stages. At these RH development proceeded normally without any obvious malformations. After the resumption of organogenesis the rate of development between controls and experimentals were similar. The difference appeared to rest mainly upon the rate of termination of diapause II.

Discussion. The annual fish *N. guentheri* is native to Kenya, Tanzania, and adjacent coastal regions of East Africa[11]. Like other annual fishes they maintain permanent populations in habitats experiencing cyclical periods of drought. It has been postulated that the species survives such an ecological catastrophe by undergoing diapause at specific stages of development[2,3]. Diapause I occurs after the complete dispersion of the amoeboid blastomeres during epiboly. Diapause II occurs prior to heart contraction and organogenesis. Diapause III occurs prior to hatching in some annual fishes and is characterized by bradycardia and cessation of yolk mobilization[2]. In *N. guentheri* the latter has not been observed. Instead, the pre-hatching embryos appear to undergo the delayed hatching phenomenon commonly observed in *Fundulus confluentus*[12].

The precarious nature of the annual fish habitat required the evolution of embryonic stages that are relatively insensitive to environmental insults. As in insects and other arthropods, diapause in annual fishes represents an intensification of the organism's resistance and serves to delay the development of sensitive structures until the return of favorable environmental conditions. The extreme susceptibility of diapause I to partial desiccation may be explained by the possibility that the 1st diapause may not exist during the major part of the dry season. Diapause I is normally induced by low oxygen tension and the presence of adult-produced inhibitory substances in the aquatic environment[3,7]. But the dissappearance of the transitory ponds during the dry season results in the elimination of the adult fishes. This event, coupled with the increasing oxygen availibility to the substrate, would contribute to the rapid

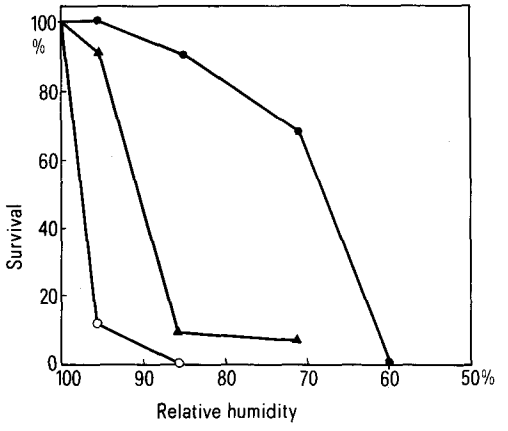

Figure 1. The survival of embryos exposed for 3 h to various controlled relative humidities. Diapause I, ○; diapause II, ●; prehatching, ▲.

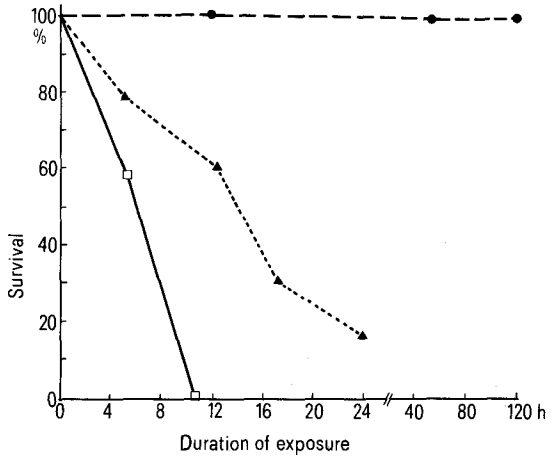

Figure 2. The survival of diapause II embryos exposed for extended periods to several controlled relative humidities. 92% RH, ●; 80% RH, ▲; 40% RH, □.

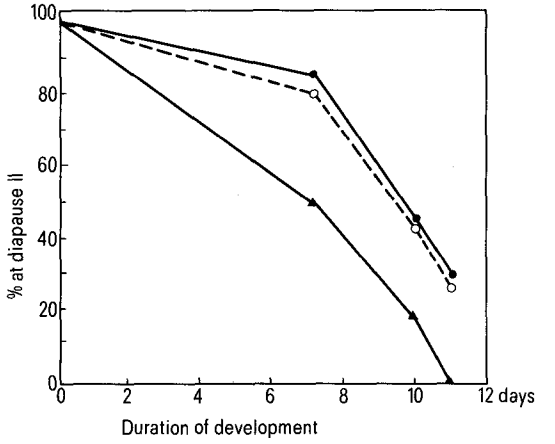

Figure 3. Rate of termination of diapause II in embryos maintained at various controlled relative humidities. 97% RH, ●; 92% RH, ○; control, ▲.

termination of diapause I. Since the inter-diapausal developmental time is less than 1 week, the embryonic population quickly enters the 2nd stage of diapause before the drought conditions become intense.

Photoperiodism is the only predictable environmental stimulus by which organisms sense the changing of the seasons. The short-day photoperiods experienced by the maternal generation during the later part of the wet season stimulates the production of embryos pre-programed to undergo diapause II[5,6]. Previous studies on the effects of extreme temperatures have shown that diapause II is the most resistant stage in annual fish ontogeny[8]. Preliminary data also indicated that the chorion of diapause II embryos are most resistant to proteolytic digestion by pronase in comparison to all other stages (unpublished). In the light of the present data on the effect of partial desiccation, it is possible that the vast majority of *N. guentheri* embryos may exist in the form of diapause II during the major part of the dry season. The retardation of diapause termination by partial desiccation may complement the photoperiod effect to assure the survival of the embryonic population until the next rainy season.

Studies under controlled laboratory conditions may not necessarily parallel what occurs in nature. Confirmation of the present hypothesis will have to wait until detailed field studies become available. The elucidation of the mechanism of annual fish survival in nature is essential since *N. guentheri* is presently being considered as a possible biological control agent of malarial mosquitoes in nature[13].

1 Bailey, R.G., Afr. J. trop. Hydrobiol. Fish. *2* (1972) 33.
2 Wourms, J.P., J. exp. Zool. *182* (1972) 389.
3 Peters, N., Int. Revue ges. Hydrolbiol. *18* (1963) 257.
4 Markofsky, J., and Matias, J.R., J. exp. Zool. *202* (1977) 49.
5 Markofsky, J., and Matias, J.R., Chronobiologia *4* (1977) 130.
6 Markofsky, J., Matias, J.R., Inglima, K., Vogelman, J.H., and Orentreich, N., J. exp. Biol. *83* (1979) 203.
7 Inglima, K., Perlmutter, A., and Markofsky, J., J. exp. Zool. *215* (1981) 23.
8 Matias, J.R., and Markofsky, J., J. exp. Zool. *204* (1978) 219.
9 Solomon, M.E., Bull. ent. Res. *42* (1951) 543.
10 Wourms, J.P., J. exp. Zool. *182* (1972) 143.
11 Jubb, R.A., Ann. Cape Prov. Mus. nat. Hist. *8* (1969) 1.
12 Harrington, R.W., Ecology *40* (1959) 430.
13 Hildemann, W.H., and Walford, R., WHO/VC/24, WHO/EBL/7 (1963).

Receptors for thymosin fraction V on rat thymic lymphocytes[1*]

Renata Brelinska[2] and J.B. Warchoł[3]

Division of Clinical Morphology, University of Ulm, D-7900 Ulm (Federal Republic of Germany), 20 July 1981

Summary. Binding by rat thymus lymphocytes of thymosin V, labeled with colloidal gold, was studied. Under the experimental conditions employed at least 2.8% cells exhibited thymosin binding sites.

Peptides of fraction V of thymosin induce expression of T cell surface antigens (Thy, Tl, Ly)[4-6] and differentiation of presursor cells into T_1, and then T_2 immunologically competent cells[7].

Although the mechanism of action of these peptides is still controversial, data from several laboratories indicate that interaction of a target cell population with thymic peptides is mediated by cyclic AMP[8]. Until now, specific surface receptors for thymic peptides have been demonstrated on the following target cell populations; receptor on T cell lymphoblastoid lines for serum thymic factor (FTS)[9], receptors for thymus factor X (TFX, peptides from calf thymus) on rat thymocytes[10].

In this report we present evidence for surface receptors of rat thymic lymphocytes for colloidal gold-labeled peptides of thymosin fraction V.

Material and methods. Thymic lymphocytes were teased from thymuses of Wistar rats, aged 36 days. The suspension of lymphocytes was centrifuged at $2000 \times g$ and washed in PBS. The cell suspension contained approximately 99% lymphocytes, of which 98% were viable, as evidenced by the trypan blue dye exclusion test. Washed cells were fixed with 2.5% glutaraldehyde in 0.5 M phosphate buffer, pH 7.3 for 15 min at 4 °C.

Colloidal gold was prepared by reducing chlorauric acid ($HAuCl_4ICN$, Merck) with trisodium citrate, according to the method described by Georghegan and Ackerman[11]. The gold particles obtained were 20 nm in size. Thymosin was added directly to colloidal gold in a dose of 0.2 mg/ml, at pH 7.4. The gold-protein complex (T-Au) was centrifuged several times to wash the colloidal gold free of traces of nonadsorbed peptides and then was diluted in PBS. Thymic lymphocytes were incubated with T-Au for 60 min at room temperature. Further processing included washing, postfixation in 1% OsO_4, dehydration and embedding in resin according to Spurr, in a routine way. Ultrathin and semithin sections were contrasted in a routine way and examined in JEOL JEM 100 C electron microscope.

The main control reaction involved preincubation of the cells with a solution of thymosin (0.2 mg/ml) prior to incubation with T-Au. For other control reactions the following media for incubation of cells were used: bovine albumin-Au complex prepared in the same way as T-Au and a solution of uncoated colloidal gold.

Results. The site of interaction of thymosin peptides, labeled with colloidal gold, and the cell surface were localized by electron microscopy. The reaction product was detected as electron dense particles on the surface of rat thymic lymphocytes (fig. 1). Occasionally encountered non-lymphoid cells (red cells, macrophages, eosinophils) remained unlabeled. Only 2.8% cells were positive as counted in electron micrographs. The intensity and distribution pattern on the surface of the cells varied markedly. In some thymic lymphocytes gold particles covered only some region of their surface, in others the label in small patches covered a large fraction of cell membrane outline. Therefore, the real percentage of labeled cells may be higher and could be estimated exactly only on analysis of serial sections or by examining smears of labeled thymocytes. Lymphocytes with a narrow rim of cytoplasm, containing scanty organelles and medium sized nuclei, rich in condensed chromatin, were most intensely labeled (fig. 2). Lymphocytes with large nuclei and prevalent euchromatin were found to bind T-Au complexes only on part of their cell surface. Small numbers of T-Au grains were noted also on cell membranes of some dividing cells. The control reactions were negative throughout.