either with bovine serum albumin (table, g) or with the $F(ab)_2$ fragments of IgG (table, e and f), suggesting that nematocysts have specific receptors on their surface for the Fc-part of immunoglobulins. The presence of N-acetyl-glucosamine or N-acetyl-galactosamine (table, h and i), both of which are known to be the binding substrates of a variety of lectins[8], did not markedly change these binding properties.

Furthermore, the Ig-binding properties of nematocysts are species-nonspecific, since a heterologous IgG will inhibit the binding of FITC-labelled 2nd antibody (table, c and d). The same experiment rules out the possibility of nonspecific FITC-binding to nematocyst membranes.

Nematocysts have been described to be negatively charged, since they are basophilic[9]. This would provide a possible explanation for the nonspecific binding of basic proteins. However, immunoglobulins exhibit isoelectric points near neutrality.

Possibly due to their acidic nature, membrane components of nematocysts have a high affinity for divalent cations which may be substituted by cobalt[10]. However, it has been shown by these authors that at least 60% of the total amount of calcium in nematocysts are tightly bound to the membrane and cannot be removed by prolonged EDTA-treatment. This corroborates the finding that EDTA does not alter the Ig-binding properties of nematocyst membranes (table, k), nor are they affected by high concentrations of calcium which may compensate an overall negative charge of the nematocysts. It is noteworthy that sponge lectins exhibit similar cation-binding properties[5].

The results of the table indicate that the Fc-part of immunoglobulin is specifically bound to the nematocyst surface. At present, we cannot prove whether the carbohydrate moiety of Ig is responsible for the binding to nematocysts, which may contain lectins or lectin-like substances. Further studies including isolation and characterization of the Ig-receptor, e.g. by affinity chromatography will help to elucidate the structure and function of particular nematocyst components.

As can be seen in the figure, the binding of fluorescent immunoglobulins provides a simple and useful technique for the demonstration of nematocysts in frozen sections or smear preparations.

1 This work was supported by the Deutsche Forschungsgemeinschaft (Ste 105/19-21).
2 I wish to thank Drs Ute Groeschel-Stewart, Darmstadt, and Helmut Schuhmacher, Bochum, for helpful discussion.
3 S. Hammarström and E.A. Kabat, Biochemistry *8*, 2696 (1969).
4 E.A. Baldo, W.H. Sawyer, R.V. Stick and G. Uhlenbruck, Biochem. J. *175*, 467 (1978).
5 H. Bretting and E.A. Kabat, Biochemistry *15*, 3228 (1976).
6 A. Forsgren and J. Sjöquist, J. Immun. *97*, 822 (1966).
7 H.G. de Couet and U. Groeschel-Stewart, in preparation.
8 S. Hammarström, L.A. Murphy, I.J. Goldstein and M.E. Etzler, Biochemistry *16*, 2750 (1977).
9 R.N. Mariscal, Coelenterate Biology. Academic Press, New York 1974.
10 Y.J. Reid, M.A. Edwards, M.J. Connock, Stain Technol. *53*, 283 (1978).
11 N. Harboe and A. Ingild, Scand. J. Immun. *2*, suppl. 1, 161 (1973).

Toxic effects of Zn^{++} and Cu^{++} on mouse blastocysts in vitro[1]

L.J. Van Winkle and A.L. Campione

Department of Biochemistry, Chicago College of Osteopathic Medicine, 1122 East 53rd Street, Chicago (Illinois 60615, USA), 21 July 1981

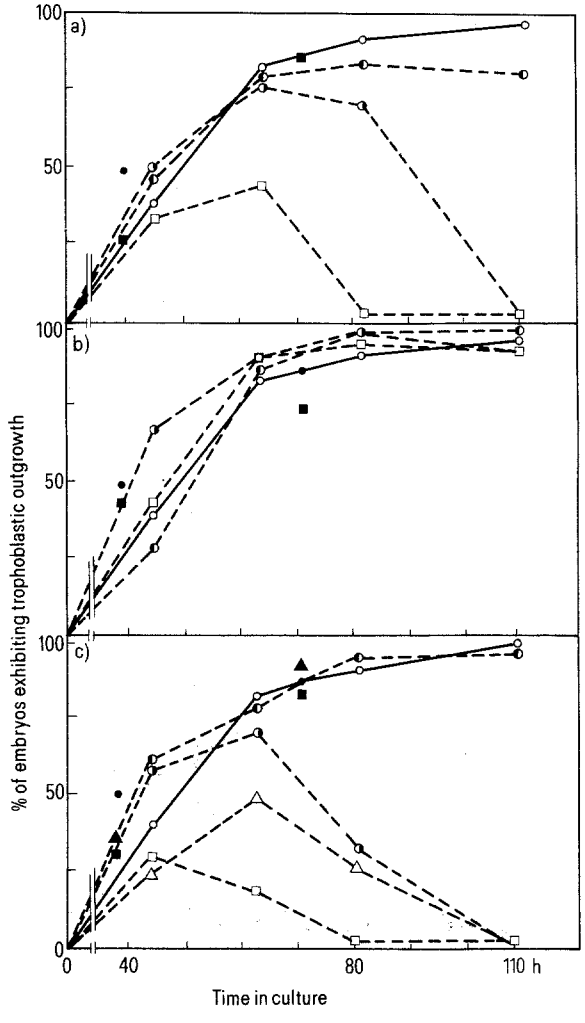
Summary. Mouse blastocysts were cultured in the presence of zinc and/or cupric chloride at various concentrations. Cupric ions were superior to Zn^{++} at inhibiting hatching of blastocysts from their zona pellucida and formation of trophoblastic outgrowths. Protein in the medium protected embryos from the toxic effects of zinc and copper.

Excess zinc ingestion by animals leads to an apparent copper deficiency while excess copper intake results in symptoms of zinc deficiency[2-7]. These toxic effects of excess zinc or copper can be ameliorated by supplementation with the 'deficient' mineral[4-7]. It has been proposed that this Zn-Cu interaction may occur at one or more of several loci including gastrointestinal or cellular absorption and incorporation of the minerals into enzymes, structural proteins and storage compounds[6,7]. I.p. injection of copper sulfate leads to an increase in zinc binding compounds in the liver of rats[8]. Thus excess copper could lead to increased zinc storage and an apparent deficiency state. Moreover, copper and zinc interfere with the intestinal absorption of each other[9,10]. However, it is not yet known whether or not competition for cellular transport sites or for binding sites on proteins, such as enzymes, contributes significantly to the antagonism between these minerals. The latter possibility could best be tested using a cell culture system so that intestinal absorption and storage of the elements would not be complicating factors.

We have used the growth and apparent survival of preimplantation mouse blastocysts in vitro to test for possible antagonism between these 2 minerals. The toxicity of cupric ions to these embryos in culture has been described[11-13] and it is well known that making intrauterine devices out of copper increases their efficacy[14,15]. Moreover, we determined that zinc can inhibit growth and development of blastocysts in vitro. Zinc wire IUD's probably interfere with pregnancy in rats by preventing implantation[15]. Nevertheless, rat uterine fluid normally contains approximately 120 μM Zn[16]. If Zn^{++} and Cu^{++} were equally toxic then the latter concentration of zinc should be toxic to embryos[11-13]. We also determined if the mere presence of Cu^{++} (100 μM) would prevent the implantation-like event of trophoblastic outgrowth in vitro as suggested by the studies of Naeslund[12].

Materials and methods. Random bred Swiss mice (8–12 weeks old) were induced to ovulate and mate with gonadotropins[17]. At approximately 87 h post coitus, blastocysts were flushed from excised uteri with Dulbecco's Modified

Eagle Medium (GIBCO) supplemented with 100 units/ml penicillin G and 50 µg/ml streptomycin sulfate (MEM). In the 1st set of experiments (table) embryos were washed, cultured[18,19] for 22–24 h in MEM then randomly divided and transferred to 1 of 11 treatment groups. 1 day later fetal calf serum (FCS; GIBCO) was added to a final concentration of 10%. After 3 more days the cultures were examined for blastocyst hatching and the formation of trophoblastic outgrowths. The pooled results of 4 independent experiments are presented (3 replicate experiments for 25 µM $CuCl_2$). In the 2nd set of experiments (fig.), blastocysts were washed, cultured for 22–24 h in MEM + 10% FCS, then randomly divided into 11 treatment groups. The MEM + 10% FCS in these treatment groups contained zero to 100 µM zinc and/or cupric chloride. Each group contained a total of 48 blastocysts and represents the combined results of 2 independent experiments. In other experiments the medium also contained 20 mg/ml bovine serum albumin (BSA; crystallized and lyophilized; Sigma Chemical Co.). In the latter cases, results of the fate of 35 blastocysts from 2 independent experiments are reported. In each experiment all Zn^{++} and Cu^{++} treatment groups were run simultaneously and observed for hatching from the zona pellucida, attachment to the culture dish and trophoblastic outgrowth. When p-values are reported, groups were compared statistically with a 2 by 2 contingency table[20] where the fraction of embryos exhibiting trophoblastic outgrowth was the parameter tested.



The effect of (a) Cu^{++}, (b) Zn^{++} or (c) Zn^{++} plus Cu^{++} on the formation of trophoblastic outgrowths by blastocysts after various time periods. The culture medium contained cupric and/or zinc chloride at the following concentrations: control (no Zn^{++} or Cu^{++}), ○; 25 µM, ◐; 50 µM, ◑; 100 µM, □; 100 µM Zn^{++} plus 50 µM Cu^{++}, △. Some cultures also contained 30 mg/ml BSA (filled symbols). When curves were close together and symbols very similar, points were offset slightly from their actual values so that they would be more discernible.

Results. Cupric ions were much more effective than zinc ions at inhibiting hatching of blastocysts from the zona pellucida and formation of trophoblastic outgrowths in the 1st set of experiments (table). 100 µM zinc reduced the fraction of blastocysts which formed outgrowths ($p < 0.05$) and all embryos appeared to expire when twice as much Zn was added to MEM. However, the final concentration of Zn in the latter case was unknown for addition of enough $ZnCl_2$ to make a 200 µM solution caused a precipitate to form in MEM. The toxicity of medium containing a zinc-induced precipitate may have been due to a high concentration of Zn^{++} and/or loss of an essential nutrient(s) to the precipitate. Embryos were not protected when zinc and copper were present simultaneously (table). In fact, 50 µM Zn^{++} appeared to act in concert with 50 µM Cu^{++} to reduce the fraction of embryos which exhibited trophoblastic outgrowth ($p < 0.01$). Embryos were unaffected when 10^{-2} M NaCl was added to the medium in addition to the NaCl already present (data not shown).

In the 2nd set of experiments, 100 µM copper did not entirely prevent implantation-like events in vitro (fig. a). The onset of trophoblastic outgrowth appeared to occur in a timely manner in the presence of 50 or 100 µM Cu^{++} after which the embryos began to disintegrate and apparently die (fig. a). When the medium contained FCS from the beginning of culture, 100 µM Zn^{++} had no detectable effect on the growth and development of blastocysts in vitro (fig. b). Nevertheless, zinc plus copper appeared to inhibit trophoblastic outgrowth to a greater extent than copper alone (fig. a vs c). BSA (30 mg/ml) protected blastocysts from the toxic effects of Cu^{++} and Zn^{++} ($p < 0.01$; fig.).

Discussion. As reported previously[11-13], cupric ions are toxic to preimplantation blastocysts. However, under culture conditions used in the present study, the mere presence of lethal amounts of copper did not prevent the implantation-like event of trophoblastic outgrowth in vitro (fig. a). Rather, it seems that a more chronic exposure to these ions

Effect of Cu^{++} and Zn^{++} on blastocyst hatching and trophoblastic outgrowth

Addition to MEM[a]	Percentage of blastocysts which were outgrowths	hatched
None (66)	75.8%	95.5%
$CuCl_2$		
100 µM (66)	0.0%	11.2%
50 µM (68)	14.7%	43.8%
25 µM (47)	70.2%	83.0%
$ZnCl_2$		
100 µM (69)	59.4%	75.7%
50 µM (67)	74.6%	92.5%
25 µM (69)	79.7%	91.3%
$ZnCl_2 + CuCl_2$		
100 µM each (71)	0.0%	28.2%
50 µM each (67)	0.0%	44.3%
25 µM each (70)	50.0%	81.4%
12.5 µM each (68)	58.8%	80.9%

[a] The number in parentheses is the total number of blastocysts cultured under the indicated condition.

finally led to the demise of all embryos including those which had formed outgrowths.

Zinc appeared to enhance rather than inhibit the toxic effect of copper on cultured mouse blastocysts. Thus, it seems unlikely that competition for cellular transport sites or for binding sites on proteins, such as enzymes, contributes significantly to Zn-Cu antagonism in animals. Instead it appears likely that Zn-Cu antagonism occurs entirely at the level of intestinal absorption[9,10] and storage in the liver[8]. It remains possible that higher levels of Zn^{++} would protect blastocysts against Cu^{++} (e.g. 200 μM $ZnCl_2$ with 50 μM $CuCl_2$). However, this could not be tested in the present studies since addition of enough $ZnCl_2$ to make a 200 μM solution in MEM produced a precipitate of unidentified composition.

It was demonstrated here and previously[13] that BSA protects blastocysts from the potentially toxic effects of cupric ions. This protein also protects these embryos against the toxic effects of copper wire in vitro[11]. The protein in 10% FCS was apparently adequate to protect blastocysts against 100 μM Zn^{++} (table vs fig. b). The high concentration of protein which prevails in the rat uterus at the time of implantation (42 mg/ml)[16] probably renders Zn^{++} (120 μM)[16] and other potentially toxic substances which may also be constituents of uterine fluid harmless. In fact, Hurley and collaborators[16,21] have argued that a diminution in the zinc concentration in uterine and oviductal fluid to about 60 μM[16] is detrimental to the development of preimplantation embryos. The full physiological significance of the relatively high concentrations of protein present in uterine fluid near the time of nidation remains to be determined. However, most previous studies of implantation-like events in vitro, where the protein concentration in the medium was an order of magnitude lower than the 42 mg/ml in uterine fluid[12,19,22-24], must now be interpreted with added caution.

1 Acknowledgment. The authors wish to thank Dr W.E. Farnsworth, Dr D.F. Mann, Mr H. Wasserlauf and Ms Barb Le Breton for their help in producing this manuscript. Supported by the Chicago College of Osteopathic Medicine.

2 J.K. Campbell and C.F. Mills, Proc. Nutr. Soc. *33*, 15A (1974).
3 H.D. Ritchie, R.W. Lueke, B.V. Baltzer, E.R. Miller, D.E. Ullrey and J.A. Hoefer, J. Nutr. *79*, 117 (1963).
4 C.H. Hill and G. Matrone, Fedn Proc. *29*, 1474 (1970).
5 N.F. Sittle and C.F. Mills, Br. J. Nutr. *20*, 135 (1966).
6 N.T. Davies, Proc. Nutr. Soc. *35*, 293 (1974).
7 N.T. Davies, Proc. Nutr. Soc. *38*, 121 (1979).
8 I. Bremner and N.T. Davies, Biochem. Soc. Trans. *2*, 425 (1974).
9 D.R. Van Campen and P.U. Scaife, J. Nutr. *91*, 473 (1967).
10 D.R. Van Campen, J. Nutr. *97*, 104 (1969).
11 R.L. Brinster and P.C. Cross, Nature *238*, 398 (1972).
12 G. Naeslund, Contraception *6*, 281 (1972).
13 M.K. Holland and I.L. Pike, J. Reprod. Fert. *53*, 335 (1978).
14 F.B. Orlans, Contraception *10*, 543 (1974).
15 C.C. Chang, H.J. Tatum and F.A. Kincl, Fert. Steril. *21*, 274 (1970).
16 D. Gallaher and L.S. Hurley, J. Nutr. *110*, 591 (1980).
17 R.E. Fowler and R.G. Edwards, J. Endocr. *15*, 374 (1957).
18 L.J. Van Winkle, Biochem. biophys. Res. Commun. *98*, 562 (1981).
19 L.J. Van Winkle, J. exp. Zool. *218*, 239 (1981).
20 C.M. Woolf, ed., in: Principles of Biometry, p.302. D. Van Nostrand, London 1968.
21 L.S. Hurley and R.E. Shrader, Nature *254*, 427 (1975).
22 A.I. Spindle and R.A. Pedersen, J. exp. Zool. *186*, 305 (1979).
23 Y-C. Hsu, Devl Biol. *68*, 453 (1979).
24 M.H. Sellens and M.I. Sherman, J. Embryol. exp. Morph. *56*, 1 (1980).

Acoustic differences between populations of western and eastern Bonelli's Warblers (*Phylloscopus bonelli*, Sylviidae)[1]

H.-W. Helb, H.-H. Bergmann and J. Martens[2]

Fachbereich Biologie der Universität Kaiserslautern, Postfach 3049, D-6750 Kaiserslautern (Federal Republic of Germany), Fachbereich Biologie der Universität Osnabrück, Postfach 4469, D-4500 Osnabrück (Federal Republic of Germany), and Institut für Zoologie der Universität Mainz, Postfach 3980, D-6500 Mainz (Federal Republic of Germany), 20 July 1981

Summary. Sonographic analyses of songs and calls of Bonelli's Warbler *(Phylloscopus bonelli)* show important structural differences between the geographically isolated western *(P.b. bonelli)* and eastern *(P.b. orientalis)* populations. Playback experiments in the field and some morphological differences suggest that these populations may be separating into 2 species.

The 2 populations of Bonelli's Warbler appear to exist as separate groups. The western subspecies (*Ph.b. bonelli* Vieillot 1819) inhabits Western Europe and the western Mediterranean basin including the Alps, Black Forest, Appenines, France, the Iberian Peninsula, and the Atlas region. The eastern subspecies (*Ph.b. orientalis* Brehm 1855) is a regular summer bird of Bulgaria and northeastern Greece while scattered populations are found in parts of southern Greece, Anatolia, Syria, and the Lebanon[3].

There are no breeding records from the Adriatic coast region including Albania and Yugoslavia (except 1 record in Hercegovina[3]). The wintering areas of the 2 subspecies are situated in the Sahel region south of the Sahara and appear to be disjunct as well[4,5].

There are small morphological differences with regard to the average wing length, relative length of primaries, and coloration[6].

The present paper describes an experimental analysis of the difference in call structure and call recognition in these 2 subspecies.

The main call note of *Ph.b. bonelli* may be characterized as a soft prolonged 'doo-éeo' differing from other *Phylloscopus* calls (e.g. *collybita, trochilus, sibilatrix*) by its descending pitch at the end (fig. 1a). It is used in contact and many arousal situations.

The different call of *Ph.b. orientalis* was first described by Reiser[7] and Peus[8] and, more recently, by Géroudet[9] and Bergmann[10]. Calls of this subspecies were recorded mainly in a breeding population north of Alexandroupolis, Thracia, Greece. There are additional records from birds presumably on migration from Cyprus, Karpathos, and the south coast of Anatolia. These calls are much shorter and harsher than the calls of the western subspecies (fig. 1b). They were represented by Reiser[7] as a 'tüp' (in German),