THE EFFECT OF SOME ENVIRONMENTAL STRESSES DURING INCUBATION ON THE EMBRYONIC AND POST-HATCH DEVELOPMENT OF CHICKENS (*Gallus gallus*) AND TURKEYS (*Meleagris gallopava*).

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ABSTRACT

Section A: Ultrasound

A series of experiments were conducted to determine the effect of ultrasound on the development of the embryo and the post-hatch growth rate of turkey poults and broiler type chicks. The intensities tested were <0.94 mW/cm.^{2}, 1.5 mW/cm.^{2}, and 28.6 mW/cm.^{2} at 2.25 MHz. and 0.94 mW/cm.^{2} at 5MHz. Exposure took place across the shell for periods of 1 to 8 minutes at 0, 1, 2, 3 or 6 days of incubation. Measurements were made on several properties of the egg albumen, egg yolk and egg shell to determine their effect on the transmission of ultrasound.

The treatment with ultrasound did not affect the development of chicken or turkey embryos as determined by hatchability, sex ratio or abnormally morphological development. The post-hatch growth rate was not significantly altered by any ultrasonic treatment.

The ultrasonic measurement studies revealed that the attenuation of ultrasound at 2MHz. across the egg shell was approximately 40dB. The attenuation (energy loss) rises with a rise in frequency, thus the loss of ultrasonic energy across the shell at 2.25 MHz and 5 MHz was probably in excess of 99.99%. Thus virtually no ultrasound reaches the embryo. Therefore, as was found in this study, no effect should be seen on the embryonic development or post-hatch growth rate.

Section B: Cold Temperature Stress

A series of experiments were conducted to study the effect of cold temperature stress during incubation on the embryonic development and the post-hatch growth rate. Commencing at 4 days of incubation eggs from broiler and W. Leghorn stock chickens were subjected to 7.2C for 24 hours. Similarly, commencing at 17 days eggs were subjected to 3.8 or 5.0C for periods ranging from 4 to 8 hours. Rates of cooling were recorded for the internal temperature of the eggs during treatments.

Exposure at 4 days of incubation to 7.2C lowered the hatchability of the broiler type eggs but not to a significant degree. However this temperature stress did significantly ($P.<05$) lower the hatchability of eggs from the W. Leghorn stock. Conversely, the broiler chicks showed a significant ($P\leq05$) increase in growth rate by 8 weeks of age due to exposure to 7.2C at 4 days whereas the W. Leghorn chicks did not. The appearance of abnormal limb development occurred in about 10% of the W. Leghorn chicks from eggs subjected to 7.2C at 4 days of incubation but none were reported from the broiler stock chicks.

Exposure to 3.8C for 4, 5 or 6 hours commencing at 17 days of incubation resulted in a significant ($P\leq.05$) reduction in hatchability, with groups exposed for 8 hours having zero hatchability in all but one trial. This temperature stress had no effect on the post-hatch growth rate of the chicks but did have some severe effects on embryonic development. Malposition of the head was noted in 50% of the embryos which failed to hatch. The hatching muscle was edematous and/or hemorrhagic in 80% of the embryos failing to hatch. Both the malpositions or the edematous-hemorrhagic hatching muscle could be the cause of the low hatchability.

Exposure at 17 days of incubation to 5.0°C for 8 hours reduced hatchability to zero. Exposure for 4 hours lowered hatchability significantly ($P<.05$) when data from all treatments were pooled. This temperature treatment had the same effect on embryonic development as 3.8°C i.e. malpositions of the head and the edematous-hemorrhagic hatching muscle. However cold temperature stress at 5°C seemed to have some effect on the growth rate of the male chicks. In one trial, the rate of growth was significantly depressed by the temperature treatment; in another it was significantly ($P\leq.05$) increased as shown by the 4 and 8 week mean body weights. In a third trial no significant difference could be detected even though the males from one temperature treatment group averaged 35 grams heavier than the other groups.

The rate of cooling of the internal egg was fairly similar for 7.2, 3.8 and 5.0°C at both 4 and 17 days of incubation. This would suggest that the embryo was still behaving as a poikilotherm at both stages. A change in the exposure methods, which was designed to allow uniform cooling of the individual egg, probably increased the mean rate of cooling per treatment group from the first trial to subsequent trials and produced an increase in mortality for the 8 hour exposure. This would suggest that the rate of cooling of the egg may be as important as the length of exposure.

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LITERATURE REVIEW

Ultrasound in Biology

The interaction between ultrasound and biological material has been studied since the 1920's (22, 23, 51). Ultrasonic waves have been accredited with the destruction of cells (11, 16, 23, 51) and animals (31, 51), and with therapeutic and curative effects in tissues and cells (17, 19, 41). Ultrasound has also been used in the diagnosis of human pregnancy (20) yet some researchers have suggested that exposure of chick embryos to ultrasound causes phenotypic and genetic changes (8 , 38, 39, 46). The use of ultrasound as a mutagenic force could prove useful in scientific research. As well, little is known of the possible effects of exposure to ultrasound waves during pregnancy diagnosis. The need for further information on the effects of ultrasound on living tissue has prompted this study.

Properties of Ultrasound

For an understanding of the biological effects of ultrasound, a basic knowledge of the physics of ultrasound and of some of its' associated phenomena is required.

Ultrasound refers to any physical vibrations which occur at frequencies greater than those detectable by the average human ear (9, 14, 17). This has been set at above 16 to 20 kilocycles per second depending upon the age of the person and other factors (9, 14, 17, 19).

The expression of frequency, the number of vibrations per given time period, was originally given in cycles per second. This has now been replaced by the term hertz, abbreviated Hz.[1], which is equal to one cycle per second. Therefore 20 KHz. (20,000 cycles per second) is approximately the ultrasonic base line.

Ultrasound behaves as an ordinary sound wave and exhibits the same basic properties. This means that ultrasound waves cannot pass through a vacuum and require a suitable transmittance medium (9, 17, 19). Ultrasound may be transmitted by gases (air), an example being a silent dog whistle (17). However the propagation of ultrasound in gases is generally limited to frequencies of less than 1 MHz due to the high attenuation (6, 17). On the other hand, propagation of ultrasound in liquids and solids is relatively easy and such media are used extensively.

Ultrasound waves generally exhibit directionality, that is they spread in a confined pattern somewhat like a flashlight beam. This occurs when the longitudinal wave is shorter in length than the dimensions of the sound source (i.e. a transducer crystal) which are perpendicular to the direction of the wave propagation. Because of the property of direction, the ultrasonic beam can be aimed at a particular target.

For ultrasonic waves, the wavelength is expressed as: $X = \frac{V}{f}$ where f is the frequency and V is the velocity of sound in the medium.

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[1]For definitions of terms see glossary, Table 1, Appendix I.

One of the most important factors in the transmission of ultrasound is the medium through which it must travel. The velocity of sound is different in different media, i.e., in air it is 343 meters/sec., in water - 1490 m/sec., and quartz - 5750 m/sec. (9). Thus for a fixed frequency, the wavelength would vary for each medium, i.e. at 1 MHz. frequency the wavelength would be 343×10^{-7}m. in air, 143×10^{-6}m. in water and 575×10^{-6}m. in quartz (9). Since the velocity of ultrasound in a specific medium is slightly altered by a change in temperature, the wavelength is also dependent upon temperature.

Another property of the medium is the attenuation (loss) of the energy of the ultrasound wave as it passes through the medium. Attenuation results from (a) absorption whereby mechanical energy is converted into heat and (b) the deviation of the energy from the parallel beam by normal reflection, refraction, diffraction and scattering (6). Absorption losses are often given for uniform media as absorption coefficients which relate loss of energy per unit length of path (6). Absorption can cause a rise in temperature of the medium exposed to ultrasound if intensities are large enough.

The specific acoustic impedance of each material can be determined. It is a function of the velocity of sound within the material and its density. Its importance arises in the coupling (transferring) of ultrasound energy (waves) from one medium to another. At the junction of the two media (called an interface) part of the ultrasound will be reflected and part will pass into the second medium. The amount of reflected energy depends upon the ratio of the two specific acoustic impedances. The greater the difference in values for impedance in each medium, the greater the amount of reflection. For example the specific acoustic impedance of water is 0.143×10^{6}, of air is 0.413×10^{2} and

reflection is nearly 100% (6), whereas water and castor oil have nearly equal impedances and very little reflection (19).

A striking phenomena of ultrasound is the occurrence of cavitation. Cavitation is the formation of a large number of small bubbles in a liquid when it is subjected to an intense sound field (14). These bubbles arise (a) from gases which were dissolved in the liquid, effervescing due to fluctuations in pressures, (b) and from the vaporization of the liquid itself due to heat of absorption and reduced pressures (19). Bubbles of the second category (b) often collapse violently as soon as formed (14, 19) while those from dissolved gases usually float to the top (2). This formation and collapse of bubbles produces very powerful disruptive forces in the media as shown by the successful use of cavitation in ultrasonic cleaning devices. Some production of heat also occurs during cavitation (18).

Production of Ultrasound

A transducer is a device for converting one form of energy into another form. In ultrasonics a transducer converts electrical energy into mechanical vibrations. The essential component of a transducer is the piezo-electric crystal (19). A piezo-electric material is one which when physically compressed exhibits an electrical potential. Conversely an electrical potential applied to such material will cause a change of shape (contraction or expansion) (17, 19). This latter property is used to create the ultrasonic vibrations.

The source of energy is called the generator. Most generators in ultrasonics are vacuum tube oscillators or transistor oscillators which produce electrical impulses which drive the transducers.

Efficient production of ultrasound waves requires a good coupling

medium, that is some material to which ultrasound waves are easily transmitted by the transducer with a minimum of loss due to reflection and absorption. The sound wave passes through the couplant to the material to be treated. Air is not a good couplant as most of the energy is reflected back. However water, paraffin, oils and special gels all act as good coupling agents.

Biological Effects of Ultrasound

Ultrasound has many effects on biological systems. One of the best ways to illustrate its' diversity and complexity is to describe some of the various biological uses of ultrasound.

Destruction of Biological Material

In 1917 Paul Langevin (31) developed a method of combining a high frequency electrical impulse generator and a piezo-electric transducer to produce ultrasonic waves in water. This has become the basis of Sonar (underwater echo detection and location system) widely recognized for its' role in submarine detection. During tests on this device he noticed that small fish in the ultrasound field were killed (31). In 1928 Wood and Loomis (51) reported a similar effect on fish and frogs exposed to a 300 KHz. field operated at 50,000 volts. Paramecium were torn apart and red blood cells were easily cytolized (51). The disruption of red blood cells was confirmed by Harvey et al. in 1928 (23), using a much lower intensity at 406 KHz. Further studies by Harvey et al. in 1928 (22) at 1.25 MHz. gave similar results for single cell animals, i.e. amoeba, and for cells in tissues. They (22) concluded most cells could be cytolyzed if subjected to sufficient ultrasonic intensity. They (24) succeeded in cytolyzing bacteria using a high

intensity and a frequency of 375 KHz. Cell disruption by ultrasound is now an established procedure (6, 17, 19) especially in biochemistry where it is used as a means of releasing enzymes from bacteria or from animal tissues such as liver. Since the main cause of cell rupture is cavitation (17, 19) lower frequencies of 20 to 100 KHz. are preferred as it requires less power to create cavitation at low frequencies.

Ultrasonic Therapy

One of the most widespread and widely accepted biological applications of ultrasound is ultrasonic therapy. Pohlman in 1938 (17) is generally credited with the first use of ultrasound for medical therapy, and by 1944 (29) clinical investigation on its therapeutic action were in progress. Ultrasound therapy was studied fairly extensively during the late 1940's and early 1950's as reviewed in 1958 by Koppen (30) and Lehmann and Krusen (32). Ultrasonic therapy has been reported to be successful in the treatment of different types of arthritis, (32, 41), neuritis and sciatica (17, 30), ulcers and abscesses (30) and scar tissues (41).

The main modes of action of ultrasonic therapy are thought to be (a) a heating effect due to absorption of the sound waves (4, 17, 30, 41) resulting in increased cellular activity and blood flow and (b) due to the physical effect of the vibration on the tissues and the cell particles, acting somewhat as an internal massage (17, 41). Ultrasound is also thought to have an unknown effect upon the nervous system (41) as it sometimes brings about relief of pain (30, 32, 41). This relief cannot be explained by heat and massage, or duplicated by alternate methods of therapy.

The usual frequencies employed in therapy range from 800 KHz. to

1 MHz. (30, 41) but have been reported as low as 300 KHz. and as high as 3 MHz. Intensities usually range from 0.25 to 5 watts/cm.^{2} (30, 32, 41). Application is usually through a water, oil, or a jel medium. A continuous wave is generally employed as pulsed beams have given any difference in effect (32).

Workers in ultrasonic therapy have a tendency to disregard frequencies as a significant factor, often omitting them in the literature (32), but they stress control of intensities (30, 32) and use this as a more important control parameter. Perhaps this is due to a desire to avoid overexposure which could result in tissue burn (41) or in cavitation which could destroy tissue cells (23, 41).

Ultrasound in Neurology

The analgesic effect of ultrasound on nerves was mentioned previously. A more important neurological application is the use of ultrasound to create reversible and irreversible lesions in neural tissue (3, 4, 11, 16, 25, 33). One of the basic means of investigation of the function of neural regions of the brain has been to create a lesion in a given area and to observe the effect on behavior or on physiological function (16, 33). By conventional methods of surgery electrodes or X-ray, additional areas other than the desired area were always destroyed. Thus it was not always certain which area contributed to the reaction. The use of focused ultrasound eliminates the destruction of intervening tissue (16, 33). A focused beam is used which may be formed by several transducers (15) or by a single focussed transducer (4, 33). A focused transducer is one with a concave crystal or a focussing device which causes the waves to concentrate their energy

in a given focal area, much as a magnifying glass can focus light waves to burn paper. The focal area can be calculated by the parameters of the transducer and medium (4, 33) and a lesion of accurately defined size in any desired position in the brain (4, 15, 33).

The frequency varies from 900 KHz. to 3 MHz. depending upon the depth to be penetrated, size of lesion desired and the intensity of application (4, 33). As a general rule the higher the frequency the smaller the lesion but size will also increase proportionately to exposure time. At higher frequencies, acoustic absorption is greater and the effective penetration depth is lessened. Both pulsed and continuous waves are used with pulsed waves giving a more spherical lesion (4, 33). Intensities are usually fairly high, varying from 48 to 160 watts/cm^2. (11) in some experiments, to higher than 1000 watts/cm^2. in others (25, 33). As intensity increases the required exposure time declines (11).

The exact cause of the lesion is not known although cavitation has been ruled out by most workers (3, 11, 16, 33). Baldes (3) and his co-workers feel that it is mainly due to local heating within the tissue but both Fry (16) and Dunn (11) have ruled out local heating as well. As the physiological response change is immediate and the histological change in cells is not, Dunn (11) feels that ultrasound somehow causes submicroscopic changes which later manifest themselves in histological changes.

The ability to create lesions in this manner is useful in neuro-anatomy, neurophysiology, psychology and in neurosurgery. W. J. Fry and his co-workers (16))have successfully treated people with Parkinsons disease by this method.

Diagnostic Uses of Ultrasound

Diagnostic ultrasound works on the echo-location principle as does Sonar. The transducer sends out a pulsed ultrasound wave through the media, in this case tissues. Since each type of tissue has a different acoustic impedance, at the interface of differing tissues some of the sound wave is reflected back to the transducer. The transducer, acting as a receiver, relays the sound wave back to the display oscilloscope as an electrical impulse base and the reflected waves show up on the screen as peaks or blotches a calibrated distance from the face of the transducer (6, 19). Refinements in this general process have led to ultrasonic scanning techniques which can produce a cross-section picture (a sonotomograph or echograph) much like an X-ray plate. Polaroid cameras are used to take a permanent record of the scan as it appears on the oscilloscope and are used for diagnostic purposes.

The range of frequencies used in diagnostic work vary from 22 KHz. used in certain bone fractures (2) to 30 MHz. used in heart-beat detection (36). However the main frequencies used are 1, 2.25 and 5.0 MHz. (2.36). The average intensities are always below those used in ultrasonic therapy (19) and are generally range from 20 to 80 milliwatts (13,20). Coupling has been by water and parrafin but is now switching to gels which are especially produced for ultrasonic diagnostic work.

Ultrasonic diagnosis was suggested and tried in the 1930's and early 1940's but the electronics for proper resolution were not advanced enough until after World War II (19). Wild and Reid (49) in 1952 showed one of the first successful attempts at ultrasonic differentiation of tissues using a 15 MHz. transducer and pulsed wave. Considerable work was done by the Japanese (36, 45) in the field of detection of

tumours in brain and in the breast.

The use of ultrasonic diagnostic technique is now common. In agriculture it is used to determine back fat thickness and loin eye area in swine (19, 48). Recently it has been used in pregnancy testing and diagnosis and in the detection of fetal heart beat (20). This last area of pregnancy scans and fetal heart beats is one in which advancement has been extremely cautious due to possible deleterious effects on the developing embryo. Although there have been no reported effect of ultrasound scans on human embryos, pregnancy scans are usually not done until 4 or 5 months at least.

Ultrasound as an Aid in Biological Research of Development and Growth

In 1928 Harvey and Loomis (23) studied the effect of ultrasound on an Elodea leaf. They described what they called "intracellular stirring"; the rapid swirling of cell contents and protoplasm without disruption of the cell wall. This intracellular stirring is now known as "streaming" (19). They (23) suggested "the interesting possibility of converting an egg with determinate cleavage into an indeterminate one by thoroughly mixing and redistributing the organ forming substances of its interior," i.e. creating a phenotypic and/or genotypic change in the embryo by use of ultrasound.

In 1928 Harvey *et al.* (22) reported some tests on embryos and fertilized eggs of *Fundulus* hiteroclitus (c.n. - killifish) at a frequency of 1.25 MHz. and unspecified intensity. For the embryos, which had a visible heart beat, the only response was an increased heart rate which they felt was due to the heat of absorption. Fertilized eggs, at a non-lethal intensity such that the cells were not cytolyzed nor the developing membranes ruptured, developed normally.

A number of reports of unusual effects of ultrasound have been reported from the field of botany. Wallace (47) in 1948 exposed growing points of 8 species of plants to ultrasound (400 KHz. at 8-10 W/cm.^{2} for from 5 to 60 seconds) and reported many plants that had different flower type and flowered earlier than normal. These traits carried on at least to the second generation.

Spencer (43, 44) reported that in peas, presoaked and then exposed to ultrasound of 20 W/cm.^{2} intensity for 30 seconds, the roots grew twice as long as controls, and by two weeks the test plants were twice as large as the controls. These traits were passed on for several generations but only by the female parent, suggesting a cytoplasmic change rather than genetic (18).

Work on barley by Johnson and Obolensky (27) using a frequency of 800 KHz., intensity of 5 W/cm.^{2} for 6 minutes exposure produced an increased rate of growth but a shorter mature plant.

In 1965, Dyer (12) reported permanent changes in mosses when single cells were exposed to low amplitude ultrasound (frequency not stated).

A. Gordon (18), in a very comprehensive review on botany and ultrasound, has listed positive growth responses for such plants as peas, beans, alfalfa, sunflowers, rice, barley, potatoes and sugar beets, whose seed had been exposed to various levels and frequencies of ultrasound. Yield increases have been reported for sugar beets, potatoes, alfalfa, cereals and sunflowers (18).

The ability of ultrasound to create phenotypic (28, 29, 23, 27, 43, 44) and genotypic (12, 43, 47) change is fairly well established although the repeatability is not consistent nor does the probability of a mutation seem any greater than that for X-ray or ultra-violet light

treatments (18). The area of genetic change has been supported by histological studies. In 1949 (34) an excellent series of photomicrographs were produced showing the effect of ultrasound on the genetic material of the cell. Numerous chromatid and chromosome breaks were recorded. This work, (35), done at 400 KHz., and other studies (18) associated cavitation with chromosomal breakage and DNA degradation. However in 1962 Hughes and Nyborg (26) demonstrated degradation of DNA by non-cavitational ultrasound. The assumption that the genetic materials recombine in mutant forms is supported by the previous reports.

In zoological studies, the effect of ultrasound on developing embryos and young is not as clear as in botany. As previously mentioned (22) no effect was reported on developing fish embryos or on fertilized eggs, except lethal cell disruption. In a report in 1953 (42) ultrasound at 1 MHz. and at non-disruptive intensity (0.3 - 0.5 watts/cm.^{2}) was focused on Drosophila embryos in the early blastoderm stage. Although many developed normally, many showed abnormalities such as loss of organs or displacement of organs.

Bell exposed frog larvae to 4 MHz. at 240 watts/cm.^{2} (cavitation) for 1 to 2 seconds (5). By varying the length of exposure, he could remove from 0 to 100 % of the outer layer of skin to a depth of one cell. This layer regrew to its normal condition within 8 days and no unusual development of the embryo was noted. Others have reported that frogs from eggs exposed to ultrasound grow faster but not larger (8, 38).

The effect of ultrasound on avian development has been studied (8, 34, 37, 38, 39, 46). Some of the early work was done on the intact egg with exposure attempted through the shell (8). No change was noted in the embryo. Vazquez (46) had similar results in 1963.

In contrast, removal of a small area of the shell giving direct exposure of embryo or blastodisc to ultrasound produced a significant number of deaths and abnormalities (8, 37). Lutz et al. (34) treated the exposed blastodisc of chickens and ducks to ultrasound at zero days of incubation. The frequencies used were 0.5, 1, and 1.5 MHz. with intensities of 0.1 to 3.5 W/cm.^{2}. Exposure time ranged up to 10 minutes. Eggs were broken out after 3 days of incubation and examined. All treatments on both duck and chicken eggs created a large percentage of abnormalities and deaths. Abnormalities occurred both in the head region and in the trunk.

In 1963 Vazquez (46) conducted a similar type of experiment. Through a small window in the shell he sonicated embryos at between 26-52 hours of incubation. This range was chosen because it was a period of rapid neural development in the head region and he wished to try and create auditory mutations. The frequency was 870 KHz; the intensities were given at 0.5 to 3 W/cm.^{2}, and exposure times were from 1 to 30 minutes. The couplant was a water bath at 37°C. with the transducer located either 1.5 cm. or 2-3 mm. from the embryo. Seven percent of the embryos were found to have abnormal development at 14 days of incubation. All abnormalities involved neural structures, particularly in the head region.

In the foregoing experiments most of the changes appear lethal. Many of the mutant embryos were dead at time of observation or from the description would not have been viable at the time of hatch. In none of the reported experiments were the treated embryos allowed to hatch and growth behaviour observed as in plants.

In 1968 Roncari (39) secured a patent for producing chickens with abnormal growth rates and size by the use of ultrasound. In this patent

the eggs were first incubated for at least twenty-four hours prior to treatment. The treatment took place in a water bath where a 2.25 MHz. transducer was held in contact with the shell (a window was not made in the shell) for periods up to 3 minutes. The egg was rotated in an attempt to ensure uniform exposure to the egg. The eggs were hatched and the chicks raised under normal conditions. The birds were reported to be 100-200 grams heavier than controls at 6 weeks and 600-1000 grams heavier at six months. This effect was reported to carry over from one generation until the next.

In a further report by Roncari (38) frequencies of 0.5, 2.25 and 5.0 MHz. were tested. Eggs were treated as above after 1 and 3 days of incubation. The machine was set at maximum amplitude but no intensities were given. Water was used as a couplant medium at temperatures of 20°C. - 22.2°C. Exposure time ranged from 15 seconds to 3 minutes. Embryonic mortality was quite high for the embryos treated with 0.5 and 5 MHz. frequencies. Those treated at 2.25 MHz. had a mortality rate similar to that of the controls. For the 2.25 MHz. group the sex ratio appeared to favour males three to one.

Unlike previous workers (8, 34, 37, 46) who found mainly abnormalities of the head and neural systems, Roncari (38) reported mainly malformation and malpositioning of the internal organs. Some abnormalities reported were atrophic lungs, enlarged liver, malposition of the heart and underdevelopment of bone. Again the treated birds are reported as 1000 grams heavier than controls. Although the age is not stated it would appear by the weight to be at maturity i.e. 3250 g. (7.2 lb.) for treated males, 2200 g. (4.8 lb.) untreated males, 2800 g. (6.1 lb.) treated females, and 1850 g. (4 lb.) untreated females.

These traits were reported to carry over to the next generation, indicating a genetic change.

Thus while there is considerable evidence for the possibility of genetic or phenotypic change due to ultrasound, much of the experimental work is inconclusive and contradictory. While mainly positive results have been quoted in this review, many reports of negative findings also exist (18) and the mutation potential of ultrasound is probably not as great as once believed.

Modes of Action of Ultrasound on Biological Material

The three main modes of action of ultrasound on biological material are: A) by mechanical vibrations, B) by production of heat and C) by induced chemical reactions (3, 18).

The initial action of an ultrasonic wave is to move the tissue and tissue particles in a back-and-forth motion at an ultrasonic frequency. This simple vibratory movement has been used in ultrasonic therapy as an internal massage and to loosen the collagen fibres in the treatment of scar tissue (41). The occurrence of streaming, the rapid circulation of cell fluids and contents within the cell, is due to mechanical vibrations and absorption differences (3, 19) and is possibly a cause of some phenotypic changes. When the mechanical force is of such intensity that it causes cavitation, cell disruption is clearly evident (17, 18, 19, 22). However cell viability can also be destroyed even when cavitation is not present as evidenced by the neurological work of Fry (16) and Dunn (11). Since they (11, 16) both ruled out heat as a cause, they concluded that it must be some mechanical breakdown of submicroscopic material in the cell. Chromosome breakages are caused by ultrasound creating cavitation (14, 35) and by the mechanical shear forces of ultrasound alone (18, 26).

The production of heat due to absorption is a second mode of action of ultrasound. This has been suggested as the cause of death of small marine animals killed in an intense ultrasonic field (51) and a possible cause of neurological lesions (3). Some of the beneficial effects of ultrasonic therapy have been attributed to the creation of heat in deep tissue (41).

The production of hydrogen peroxide, the oxidation and reduction of chemicals, and the increase in reaction rates of chemical process have all been induced by the presence of ultrasound (17, 18). These processes could occur within the cell with a stimulatory or detrimental effect. However most of these reactions take place in the presence of cavitation, and cavitation per se has generally been a destructive force on biological material except for the experiments on the ultrasonic treatment of seeds. Biochemical reaction rates as measured by enzyme activities have also been affected by exposure to ultrasound, both above and below cavitation intensities (18). However the results are not constant as an enzyme's activity may be increased in one experiment and decreased in another (18). The stimulation of hormone activity is another biochemical change which ultrasound has been thought to cause (8, 18, 28). Many of the growth responses reported earlier bear a resemblance to those caused by hormones. In young rats exposure of the ovaries to ultrasound (less than 2 $W/cm.^2$) over an extended period of time caused earlier sexual maturity and an increased rate of gain in body weight (28). As the offspring from these rats grew normally, a stimulation of either hormone production or hormone activity appears the logical cause. Hormone stimulation has also been suggested as the cause of increased growth rate in frogs from eggs exposed to ultrasound

(8). The means by which ultrasound induces the stimulation on enzymes and hormones has not been explained.

In lieu of the reports by Roncari (38, 39) it was decided to test the effect on the embryo of ultrasonic exposure through the intact shell. The importance of the genetic potential which could possibly be realized was one reason. A second reason was the safety factor in the diagnostic uses of ultrasound. The machine which Roncari (38, 39) reported using is of the type and frequencies, 0.5, 2.25, and 5.0 MHz., which are commonly used for ultrasonic pregnancy scans (20). However, he (38, 39) did not report the intensities from his transducers and therefore an exact comparison can not be made with intensities used in diagnostic work. Although there are no reports of ultrasonic scans during pregnancy having any effect upon the embryo (20) it was felt that further work in this regard would be very valuable.

METHODS

General Materials and Methods

In all experiments, except Number 2, eggs produced by the same broiler strain were used. Eggs of broiler stock were chosen as the growth rate of such chicks is rapid and any change should show up quickly. In Experiment 2, eggs from a flock of large white turkeys were used to see if a difference could be found in species.

All eggs were candled for cracks, doubleyolks, and other irregularities before incubation. Chicken eggs were incubated for 22 days to allow extra time in case development was slowed when eggs were removed for treatment. The turkey eggs were also incubated an extra day for the same reason. All eggs which failed to hatch were broken open and examined for fertility, age of embryonic mortality and abnormality of the embryo or the egg contents (7, 40).

All chicks and poults were wing banded at hatching and records were kept of hatchability and mortality. The birds were weighted individually at the time of hatch and then weekly to four weeks of age unless otherwise stated. All chicks and poults were sexed by professional sexers at the time of hatch except in Experiment 1.

The broiler chicks were raised in wire floored, multi-tiered, brooding batteries on a commercial chick starter ration. The turkey poults were raised in floor pens on a commercial turkey poult ration. The toms and the hens were reared separately.

Data were analysed on an Olivetti Underwood Programma 101 desk-top

computer using program cards. Experiment 1 was analysed by 2-way analysis of variance, while Experiments 2, 3, 4, and 5 were treated as one-way analysis.

Two different diagnostic ultrasound machines were tested. Both machines were capable of driving a 2.25 MHz. transducer, and had adjustable amplitude controls which could vary transducer intensities. The transducers for both machines were non-focused, portable (hand-held) types for which the energy output (watts) could be easily measured by a radiation balance.

Experiment 1

The first machine tested was a Sono-Medic, model 200U. The maximum and minimum amplitude settings were used. The manufacturers specifications rated the input to the transducer at 0.5 and 0.1 Watts/ $cm.^2$ for the high and low settings respectively.

A 2.25 MHz. non-focused transducer was used and radiation pressure measurements were made at the high and low amplitudes.

Two hundred and forty eggs were randomly set in trays for incubation. Forty-eight eggs were randomly removed per day after 0 and 1 day of incubation and allotted to treatments as in Table 1. Only eggs which appeared fertile on candling were chosen for treatment which took place after 3 and 6 days of incubation. This was done since the study of effect of ultrasound on the live embryo was of greater importance than complete randomization of treatments.

The experiment was divided and analyzed as four sub-groups according to the number of days of incubation before treatment as per Table 1. Each subgroup contained two control and 6 treatments which were determined by the amplitude (high or low) and by length of exposure (2, 4, or

8 minutes). Each treatment contained six eggs. The low number of eggs was due to the treatment procedure as only one egg at a time could be treated. Thus the actual treatment time was nearly 4 hours. Add to this the time required to allow the eggs to equilibriate to the horizontal position, to remove and retray the eggs in incubation trays, to mark the eggs, to lift the eggs in and out of the treatment media and the total time required was around 7 hours. If the eggs were kept out of the incubator much longer this effect may create a complicating factor.

Air is a poor conductor of ultrasonic waves and it also gives nearly a 100% reflection of energy when an ultrasonic wave attempts to pass from a liquid media into air. For these reasons treatment could not take place through the air cell (large) end of the egg and therefore the transducer had to be applied to the side of the egg. After removal from the incubator the eggs were set out with their long axis horizontal and left in this position until treatment was completed. This allowed the blastodisc or embryo to rotate to the top of the yolk as shown in Figure 1. In all experiments the eggs were in this position at least twenty minutes to one-half hour before treatment to ensure that the blastodisc or embryo was centered. The eggs were carefully moved in this position to the treatment medium with a minimum of disturbance. This procedure was followed for all experiments.

The couplant medium was distilled water at 30°C., and the treatment took place in a large stainless steel thermostatically controlled water bath. During treatment the transducer was held in contact with the shell (Figure 1). For one half of the exposure time the transducer was held over the center of the yolk, and for the remaining time it was moved to the four points surrounding the center, as indicated in diagram 2. A

stop-clock was used to time all treatments.

Experiment 2

The Sono-Medic generator was used at maximum amplitude to drive a 2.25 MHz. and a 5 MHz. transducer. The 5 MHz. transducer was included to see if it produced detrimental effects as described by Roncari (38). Radiation pressure measurements were made on the 5 MHz. transducer.

Two hundred eggs from a flock of large white turkeys were set in incubation trays. Eggs were drawn at random after 1 and 3 days of incubation and treated according to Table 3. There were two replicates with ten eggs per replicate, thus allowing statistical analysis of hatchability data. Exposure was for either 2 or 3 min. using either the 2.25 MHz. transducer or the 5 MHz. transducer. The couplant medium, the method of application and the treatment in water bath were the same as in Experiment 1.

Experiment 3

The ultrasonic equipment and transducer were those used in Experiment 2.

One hundred and sixty broiler stock eggs were set into incubation trays. Eggs were removed randomly for treatment after 1 and 3 days of incubation, as outlined in Table 4. Two media were compared in this experiment. One medium was distilled water (20 - 22°C.) while a mixture of one part milk to 3 parts sour cream (21 - 22°C.) composed the second medium. The sour cream was used in an attempt to create a medium resembling more closely that of the internal contents of the egg. The 2.25 MHz. transducer was used in both water and sour cream, but the 5 MHz. was used only in distilled water. All eggs were treated for 3 minutes.

In an attempt to simulate more closely the conditions used by Roncari (38, 39), the temperature of the couplant medium was changed to 20 - 22°C. Also during treatment the egg was held in a glass tubing holder 4 cm. from the bottom of a 600 ml. beaker. Other treatment methods were the same as for Experiments 1 and 2.

Experiment 4

The Sono-Medic generator at maximum amplitude was used with the 2.25 MHz. and 5 MHz. transducers.

One hundred and eighty eggs were set into incubator trays. Eggs were taken randomly after 1, 2, and 3 days of incubation and treated as per Table 5. Each egg was treated for 3 minutes, and each treatment consisted of two replicates of 15 eggs. The 2.25 MHz. transducer was used after 1 and 3 days of incubation and the 5 MHz. transducer was used after 2 and 3 days of incubation.

The couplant media was the sour cream mixture in the 600 ml. beaker at 20 - 22°C., described in Experiment 3.

Experiment 5

The second diagnostic machine, a Hoffrel Ultrasonoscope - model 101, was used for this experiment. This machine had an adjustable amplitude which was calibrated in terms of volt-pulses. Two amplitude settings were used. The first was a 400 volt-pulse, the range recommended by the instruction book for most diagnostic work. The second setting was a 1600 volt-pulse, the highest setting on the machine which was used to determine the effect of increases intensity. The transducer, a 2.25 MHz. non-focused portable type, was measured for radiation pressures at both amplitudes used.

Ten dozen broiler stock eggs were set and randomly assigned to treatments as outlined in Table 6. The treatment took place after 1 day of incubation. Each treatment contained 2 replicates of 15 eggs in each to permit hatchability and sex ratio analysis. Eggs treated with ultrasound were exposed for 3 minutes to either the low setting (400 volt-pulse) or the high setting (1600 volt-pulse). As the eggs to be treated had to be transported in an egg case in summer weather, a round trip of 14 miles, one set of controls travelled with the treatment eggs while one set of controls remained in the incubator. They are referred to as the travelling and stationary control respectively.

The method of application of ultrasound was changed slightly for this experiment. A special gel designed as an ultrasonic medium (at a temperature of 24.5°C.) replaced distilled water as the couplant medium. The egg was placed with the long axis horizontal as in Figure 1. However the transducer was not rotated but remained fixed over the center of the egg throughout the entire exposure time.

Ultrasonic Measurements

Two types of ultrasonic measurements were conducted as additional aids to interpret the effects of ultrasound on biological material.

The first series of experiments were done to obtain intensity values at the surface of the transducer. A radiation balance manufactured by Friston Electronics, Edinburgh, was used to determine the radiation pressure according to the method described by Gordon (19). Radiation pressure is a measurement, in watts, of the average power output from the transducer. The radiation pressure can be converted to the intensity if the size of the crystal in the transducer

is known. Measurements were made on the following: (a) The Sono Media generator, using the 2.25 MHz. transducer at the high and low amplitude and the 5 MHz. transducer at the high amplitude and the (b) Hoffrel Ultrasonoscope with a 2.25 MHz. transducer at the 400 and 1600 volt-pulse settings. The Sono Media generator transducer was measured in distilled, non-degassed water at 22.5°C., while the Hoffrel Ultrasonoscope with the 2.25 MHz. transducer was measured at 24°C.

The following measurements were done by M. Friese[1] with the use of his facilities in the Freshwater Institute laboratories: the velocity of ultrasound at 5.3 MHz., 20.5°C. (69°F.), in egg albumin and egg yolk; the specific gravity (density) for the above; and a direct measurement of the reflection of ultrasound from the egg shell was attempted. The absorption coefficient of the egg yolk was determined and attenuation measurements, across the entire egg and across the egg shell were made when attempts to measure the velocity of sound in egg shell failed.

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[1] M. Friese, Ultrasonic Department, Freshwater Institute, Winnipeg, Manitoba

RESULTS AND DISCUSSION

Experiment 1 - Sono-Medic generator with 2.25 MHz. Transducer.

Per cent hatchability of fertile eggs, mean hatch weight and mean 4 week body weight for each treatment are presented in Table 2. The treatments are arranged in groups according to the number of days of incubation before the ultrasonic treatment. As each incubation stage contains its own control, a simple two-way analysis of variance was performed on each group. Since there were no replicates, only one hatchability value could be obtained per treatment and therefore statistical analysis was not possible. However, there appears to be little difference between treatments within any one incubation stage, or between different stages of incubation. The range is from 50 to 100% but due to the limited number of eggs per treatment, a loss of 3 out of 6 fertile eggs represents a 50% hatch failure.

Within any stage of incubation (i.e. those treated after 0 days incubation), there were no significant differences (P .05) in hatch weights or 4 week body weights. Between stages of incubation there appears to be a slightly greater gain in weight at 4 weeks for chicks from eggs treated after 1 day of incubation, over treatment at all other incubation stages. However, this is also true for the controls and hence cannot be attributed to the ultrasound exposure.

Examination of the eggs which failed to hatch revealed that most of the embryos died during either the first 8 days or the last 4 days

of incubation, which is the normal embryonic mortality pattern (40). There were 4 abnormally developed embryos in the 187 fertile eggs, 1 from each incubation stage. All four embryos were found in treated eggs, 3 in eggs treated for 4 minutes and 1 in the 8 minutes exposure. Three of the four abnormalities were failure of the craniums to form normally associated with the absence of eyes, crossed beak and exposure of the upper spinal column. The fourth embryo had a deformed leg. The prevailing rate of abnormalities is not known for this strain.

There were two drawbacks to this experiment. One was the low number of chicks hatched per treatment, ranging from 2 to 6, and the second was that the chicks were not sexed. Generally males of this broiler strain were 50-60 g. heavier than the females at four weeks (Tables 4 and 6). In a treatment with only 3 birds it was easily possible (probability of 1/8) to have all males or all females. Thus treatments with large average weights could have been due to the fact that all the birds were males. The smallness of numbers was due to the length of time required to treat the eggs as discussed in Methods. In subsequent experiments the problem of low numbers was relieved by decreasing the exposure time and by using fewer treatments. The problem of possible weight difference due to sex was eliminated by sexing at one day of age but it resulted in a small number per treatment-sex group.

Experiment 2

The results of the same type of ultrasound treatment (as in Experiment 1) on turkey embryos are presented in Table 3.

The per cent hatchability was not significantly different among treatments ($P.05$). Hatchability was analyzed by a one-way analysis of the arcsine transformation of the percentage hatch of fertile eggs.

This method was also used for hatchability analysis in Experiments 3, 4 and 5.

Examination of embryos which failed to hatch revealed the normal pattern of embryonic mortality. There were three abnormal embryos out of 161 fertile eggs. The one found in the control eggs had no intestinal organ differentiation, merely a large open sac. The two other abnormalities, from treated eggs had deformed legs, and one of these had a humped back.

The sex ratio was analyzed by performing a one-way analysis on the arcsine transformation of the percent females per replicate, but no significant difference (P .05) was found. The total number of females was 50 out of 100 poults hatched.

The body weights at hatching, at 4 weeks and at 8 weeks were not significantly different among treatments. (P .05).

Experiment 3

The results of treating chicken embryos after 1 and 3 days of incubation with ultrasound, using 2.25 MHz. and 5 MHz. transducers, are summarised in Table 4.

A significant difference (P .05), due to treatment, could not be detected for per cent hatchability. There was a slight drop in hatchability, when the sour cream medium was used which was shown also in Experiment 4.

Examination of dead embryos showed a normal distribution for age of death with no increase associated with treatment.

Abnormally developed embryos numbered 3 out of 131 fertile eggs. All occurred within treated groups. The 2.25 MHz. - sour cream medium group, treated after 1 day of incubation contained one embryo with

abnormally short limbs. The 5 MHz. - water medium group, treated after 1 day of incubation had one embryo with a humped back and incompletely formed digestive tract and internal organs. The third deformity, which consisted of short curly wing feathers and down, had been treated with 2.25 MHz. transducer in water after 3 days of incubation. The presence of large bacterial masses in the eggs in which embryonic mortality occurred by the 8th day was markedly higher in this experiment than in previous ones. This was especially so for eggs treated in sour cream.

No significant change in the sex ratio was evident (P .05) due to treatments although the number of females hatched (62) was slightly higher than males (50). Analysis of body weight at time of hatch and at 4 weeks showed no significant differences due to treatment (P .05).

Experiment 4

The summary of the results of eggs exposed to 2.25 and 5 MHz. frequencies in a sour cream medium is given in Table 5.

Hatchability had to be calculated on the basis of eggs set rather than fertile eggs for this experiment. This was due to the fact that the bacterial infection in the eggs which were either infertile or had early dead embryos (0-8 days) was so extensive that for 50 percent of the eggs the embryo had almost completely disintegrated and could not be distinguished from bacterial growth. These eggs ranged in appearance from those with a whitish mass covering the yolk, a pinkish tinge to the albumin and a slightly offensive odor, to those in which the only contents were a greyish fluid (Black Rot) with an extremely offensive odor. A bacteriological culture study of these eggs showed contamination with cultures of E. coli, B. subtilus, and H. streptococcus in all treatments including controls.

Although there was no significant differences in hatchability (P .05) between the treated and the control eggs, hatchability for this entire experiment was considerably lower than all of the other experiments using the same source of hatching eggs. Therefore it was felt that the reduction in hatch was not due to infertile eggs, but due to the massive bacterial infection. In Experiment 3 it was noticed that the sour cream treated group had a lower hatchability and a heavier bacterial infection than the other groups. Thus the source of the bacterial infection probably was the sour cream and the contamination then spread to the control eggs as they were set in the same incubation trays. However, the use of ultrasound may have aided the bacterial infection as some workers (10, 50) have reported an increased bacterial content in eggs in cleaned in ultrasonic cleaners. Therefore, although some ultrasonic cleaners have been patented (1) their usefulness, especially with hatching eggs, is somewhat dubious due to a possible increased bacterial contamination.

Examination of embryos failing to hatch was complicated by the extreme bacterial infection as previously discussed. Of the embryos which died in the later stages of incubation, 4 abnormalities were recorded for 150 eggs set. In the controls, one embryo had curly, frizzled feathers and down. A second control embryo had a badly twisted leg which appeared to have been caused by being wrapped up in the umbilical cord. The umbilical cord was also wrapped around the neck and hence strangulation likely caused death. One embryo in the 5 MHz. - 3rd day treatment group was in the same condition as the above described bird, while the fourth abnormality, also from the 5 MHz. - 3rd day treatment group, had frizzled feathers and down similar to the first embryo described in the control group.

No significant differences occurred in the sex ratio (P .05) and the total number of females hatched was 1 less than the number of males.

There was no significant difference in hatch weight due to treatment (P .05). However the average hatch weight of chicks in this experiment is considerably lower than the previous ones (36 gm. in Experiment 4 as compared with 41 gm. in Experiment 1 and 45 gm. in Experiment 3). This hatching weight difference is likely due to the difference in size of eggs set, which were noticeably smaller (16 eggs could be trayed per row whereas only 15 eggs could be put in the previous Experiment). It is also possible that bacterial infection, resulting in decomposition of some of the egg contents, had some effect on the hatch weight.

There was no significant differences among the treatments (P .05) for average body weight at 4 weeks. However the average 4 week body weights for this experiment are lower than those in other experiments (423 g. for males in Experiment 4 versus 575 g. and 593 g. for males in Experiments 5 and 3 respectively). The high bacterial infection may account for this as the chicks appeared weak and sick at hatch and for the first week. Mortality was slightly higher in this experiment than in the others (4.5% for Experiment 4 versus 1.7, 1.8, 1.8% for Experiments 3, 5 and 1, respectively.

Experiment 5

The results of treatment of eggs with the Hoffrel generator and 2.25 MHz. transducer are summarized in Table 6.

Neither the travelling condition nor the ultrasound exposures resulted in any effect upon hatchability or sex ratio. Fifty-five chicks of each sex were hatched. Hatchability was extremely high and

only 10 out of 120 eggs set failed to hatch. Of these, 5 eggs were infertile, one died at 3 days and 4 died at 17-21 days of age. No abnormally developed embryos were found. Mean body weights per treatment were not significantly different (P .05) at time of hatch, at 4 weeks or at 5 weeks of age.

Ultrasonic Measurements

The radiation pressures and the ultrasound intensities at the face of the transducers are presented in Table 7. A radiation pressure measurement could not be obtained for the 2.25 MHz. transducer and the Sono-Medic generator even with the Friston Intensity meter at maximum sensitivity. However the oscilloscope patterns indicated that some output did occur. Therefore the radiation pressure was given as less than 2.69 mW., the minimum level detectable by this intensity meter which as an intensity value was < 0.94 mW/cm.^{2} The 5 MHz. transducer was measured at 2.7 mW. giving an intensity value of 0.949 mW/cm.^{2} when used with the Sono-Medic generator.

For the 2.25 MHz. transducer driven by the Hoffrel Ultrasonoscope the intensities were 28.6 mW/cm.^{2} at the high amplitude (1600 volt-pulse) and 1.5 mW/cm.^{2} at the low setting (400 volt-pulse).

The results of the ultrasonic measurements done in the laboratories of the Freshwater Institute under the direction of M. Friese are presented in Table 12. The velocity of sound at 5.3 MHz. could not be resolved for egg shell nor could a reflection value be obtained directly.

The attenuation across the entire egg for ultrasound at 2 MHz. varied from 72 decibels (dB) when the transducer was in contact with the shell to 80 dB when there was no contact between the egg shell and transducer. Across the single shell thickness attenuation was approximately

40 dB, which means only 1/10,000 (0.01%) of the original energy output from the transducer was crossing the shell. By using a special ultrasound analyzer, it was determined that as the frequency declined the amount of energy that crossed the shell increased. Due to the magnitude of the attenuation across the egg shell alone, the losses due to absorption and reflection by the egg white and egg yolk were negligible, and were not calculated. As the frequencies used for the treatment of eggs in Experiments 1 to 5 were all greater than 2 MHz., the attenuation in those experiments would be even greater than reported above.

At the intensities and frequencies used in these experiments no ultrasound energy was reaching the embryo in the egg due to the attenuation across the shell. This could account for the fact that there was no differences in growth or development, as reported by other workers (8, 38, 46), caused by ultrasonic treatment in the experiments done in this study. This also substantiates the observations of Brettsneider (8) and Vazquez (46) who could not obtain a significant number of mutations when treatment was attempted through the shell and who concluded that the shell afforded a protective shield against ultrasound waves. It would also throw doubts on the work of Roncari (38, 39). At an attenuation of 40 dB, in order to pass an appreciable amount of energy across the shell, the intensities would have to be of the order to cause very violent cavitation and this was not indicated in his (38, 39) reports.

In order to overcome the problem of shielding by the shell, the embryo must be exposed to the ultrasound through a window cut in the egg shell in a manner similar to that reported by Lutz et al., (34) Brettsneider (8) and Vazquez (46). This operation would increase greatly

the time required to complete a treatment and increase mortality, but would insure exposure of embryo to ultrasound.

Gordon (21) has suggested that a more positive approach would be to determine a non-cavitational exposure level of ultrasound that was lethal. Working back from this level it may be possible to find a treatment which could create a viable beneficial mutation. However, the available equipment is too limited for the proposed investigations. In the experiments by Vazquez (46) and Lutz et al. (34) the acoustic intensities reported were from 0.5 to 3.0 W/cm.^{2}. The highest intensity from the available diagnostic machines was 0.028 W/cm.^{2}, a value approximately 1/20 that of the lowest mutagenic level reported by Vazquez (46). In order to increase the intensity, Gordon (21) recommended using a higher amplitude generator capable of 100 Watts output to transducer, and improving the efficiency of the transducer by using one with an air back design. The minimum efficiency of the transducer would be 30%, giving 30+ Watts power output. If a 2 cm. transducer was used this could give an intensity up to at least 9.5 W/cm.^{2} at the face of the transducer.

Because of the cost a single operating frequency was suggested by Gordon (21) either 1 MHz. or 2.25 MHz. However one could use a range of frequencies with the proposed machine by changing transducers and making minor adjustments to the generator. It was also felt that pulse duration and repetition rates should be adjustable to give more flexibility in the experiments. With this type of machine the effects of variations in intensity, frequency pulse width and repetition rates could be studied as well as the biological factors.

However, two factors prevented continuation of this project. The

first, was the time required to obtain new equipment and carry out sufficient investigations. It would have taken at least 4 months to obtain the new equipment of the type required. Then it would have taken, with the aid of two technicians, three to six months after arrival of equipment to conduct a sufficient number of experiments. This would make it almost a year before completion of experimental work. The second factor was that adequate technical advice and guidance from a person with a thorough knowledge of ultrasound was not available. This type of project should be done jointly with a full-time specialist in ultrasonics. Due to these factors the project was terminated at this point.

SUMMARY AND CONCLUSIONS

The treatment of chicken and turkey embryos with ultrasound propagated across the shell was studied to determine the effect on embryonic development, sex ratio, and post-hatching growth response.

Two sources of ultrasound with the following characteristics were employed:

(A) Sono-Medic-200U generator with a 2.25 MHz. at intensity of <0.94 mW/cm.^{2} or a 5 MHz. transducer at 0.949 mW/cm.^{2} intensity.

(B) Hoffrel Ultrasonoscope-101-generator with a 2.25 MHz. transducer and high and low amplitude settings giving intensities of 28.6 mW/cm.^{2} and 1.5 mW/cm.^{2} respectively.

Using the Sono-Medic generator, chicken eggs were treated after 0, 1, 3, or 6 days of incubation for 1, 2, 3, 4 or 8 minutes. Turkey eggs received treatment for 1 or 3 minutes after 1 or 3 days of incubation. The Hoffrel machine was used to treat chicken eggs for 3 minutes after one day of incubation.

Three types of couplant media used were distilled water, sour cream and a special jel designed for use in ultrasound diagnostic work. Measurements were done to determine the attenuation of ultrasound energy in egg shell, egg albumin and egg yolk and the intensities output of the transducers.

The Sono-Medic generator combined with a 2.25 MHz. or a 5 MHz. frequencies transducer at intensities of <0.94 mW/cm.^{2} and 0.949 mW/cm.^{2},

respectively did not produce any effect on post hatching growth rate:

(A) in chickens from eggs receiving the following treatments:

(a) 2.25 MHz. treatment after 0, 1, 3 and 6 days of incubation when exposed for 2, 4 or 8 minutes in water medium.

(b) 2.25 MHz. treatment after 1 and 3 days of incubation, exposure for 3 minutes with a couplant media of water or sour cream.

(c) 5 MHz. treatment after 1 and 3 days of incubation, exposure for 3 minutes in water couplant medium.

(d) 2.25 MHz. treatment after 1 and 3 days incubation, 3 minutes exposure, sour cream medium.

(e) 5 MHz. treatment after 1 and 3 days of incubation, 3 minutes exposure, sour cream medium.

(B) in turkeys from eggs receiving the following treatments:

(a) 2.25 MHz., treatment after 1 and 3 days incubation, for 1 or 3 minutes exposure in water medium.

(b) 5 MHz., treatment after 1 and 3 days incubation for 1 or 3 minutes exposure in water medium.

Hatchability and sex ratio were not affected in chickens or turkeys for the above conditions by the use of ultrasound.

The Hoffrel Ultrasonoscope combined with a 2.25 MHz. transducer and producing ultrasound at 28.6 mW/cm.^{2} and 1.5 mW/cm.^{2} produced no effect on post hatching body weight, hatchability or sex ratio in embryos from eggs treated at these intensities for 3 minutes after 1 day of incubation. Neither embryonic mortality nor post hatching mortality

appeared to be affected by the ultrasonic treatment of embryos.

The occurrence of abnormal embryos did not appear appreciably higher in the embryos exposed to ultrasound than for the controls. In chicken eggs, 2.05% of the total treated embryos were deformed while 1.53% of the total control embryos were deformed. The prevailing rate of spontaneous occurrence of abnormalities is not known for this strain but would likely fall within this range.

The dipping eggs into sour cream appears to be detrimental to hatchability and growth due to heavy bacterial infection.

The attenuation by the egg shell of ultrasound at 2 MHz. is approximately 40 dB (i.e. a 99.99% energy loss). The attenuation appears to rise with frequency so that the loss at 2.25 MHz. and 5 MHz. would be even greater.

The passage of ultrasound energy through the egg shell at intensities less than the cavitation threshold and for frequencies higher than 1 MHz. is virtually nil due to the magnitude of the attenuation by the egg shell.

The inability of the ultrasonic energy to reach the embryo effectively explains why no changes were noticed for hatchability, sex rates or growth rate.

The only effective means of treating the embryo would be by removal of a portion of the egg shell as reported by Vazquez (46) Lutz (34) and Brettsneider (8).

Table I

Treatment of fertile chicken eggs[1] with ultra sound at 2.25 MHz. frequency in a water couplant media at 30°C.

Number of days Incubation	Amplitude		Length of Treatment (minutes)			
0	High	0.94 mW/cm.^{2}	0	2	4	8
0	Low	0.94 mW/cm.^{2}	0	2	4	8
1	High	0.94 mW/cm.^{2}	0	2	4	8
1	Low	0.94 mW/cm.^{2}	0	2	4	8
3	High	0.94 mW/cm.^{2}	0	2	4	8
3	Low	0.94 mW/cm.^{2}	0	2	4	8
6	High	0.94 mW/cm.^{2}	0	2	4	8
6	Low	0.94 mW/cm.^{2}	0	2	4	8

[1] six eggs were used per treatment.

Table 2

The effect of ultrasound treatment through the shell on 0, 1, 3 or 6, day old chicken embryos with respect to hatchability and mean body weight using a 2.25 MHz. transducer at < 0.94 mW/cm.^{2} for 0, 2, 4 or 8 minutes in water couplant.

	Amplitude Setting	Length of Ultrasound treatment after 0 days Incubation[1] 0 min.	2 min.	4 min.	8 min.	Length of Ultrasound treatment after 1 day Incubation[1] 0 min.	2 min.	4 min	8 min.
Percent Hatch of Fert.	High[2]	83	60	66	83	83	100	66.6	83
	Low[2]	80	83	100	83	83	83	83	100
Mean Hatch Wt-g.	High	40.8	41.6	39.3	40.2	41.4	40.6	41.5	38
	Low	40.8	44.2	41.1	42.2	40.5	39.6	39.8	42.1
Mean 4-wk body weight g.	High	637	628	573	649	665	677	665	640
	Low	608	567	656	599	654	678	659	654

		after 3 days Incubation[1] 0 min.	2 min.	4 min.	8 min.	after 6 days Incubation[1] 0 min.	2 min.	4 min.	8 min.
Percent of fertile hatch	High	100	66	83	66	66	83	100	83
	Low	100	100	80	80	80	100	100	83
Mean Hatch weight g.	High	41.3	43.0	40.2	42.7	39.6	41.4	41.5	45.4
	Low	39.0	41.0	40.7	41.8	42.0	41.1	42.5	42.4
Mean 4-wk body weight g.	High	598	707	621	629	604	640	681	596
	Low	662	634	632	572	647	596	606	623

[1] Hatch weights and 4 week body weights were not significantly (P.05) different within incubation periods. Hatchability could not be analysed statistically.

[2] See Table 7 for intensities values at high and low amplitude settings.

Table 3

The effect of ultrasound treatment through the shell on 1 or 3 day old turkey embryos with respect to hatchability, sex ratio and mean body weight using a 2.25 or 5.0 MHz. transducer at < 0.94 mW/cm.^{2} and 0.94 mW/cm.^{2}, respectively, for 0, 1 or 3 minutes in water couplant.

Treatments[1]

Days of Incu. before treat.	Frequency in MHz	Length Exposure in Minutes	Hatchability[2] Rep. 1	Hatchability[2] Rep. 2	% Females[2] Rep. 1	% Females[2] Rep. 2	Mean Weights[3] in grams: Hatch Males	Hatch Females	4 weeks Males	4 weeks Females	8 weeks Males	8 weeks Females
0	0	0	66	66	75	66	58.3	58.4	1090	909	3147	2580
1	5	1	77	62	42	0	56.1	54.0	1031	919	3098	2485
1	5	3	87	85	57	83	56.5	56.4	1078	947	3234	2622
1	2.25	1	57	66	50	33	58.8	56.5	979	959	2890	2632
1	2.25	3	71	87	60	85	61.0	54.4	1112	955	3359	2744
3	5	1	66	20	50	100	56.5	57.0	1089	998	3259	2905
3	5	3	85	75	33	33	54.6	55.2	1088	964	3152	2882
3	2.25	1	75	88	33	62	59.7	54.7	1099	917	3233	2646
3	2.25	3	66	40	16	50	57.3	54.0	1070	930	3095	2565

[1]Each treatment started with two replicates of ten eggs

[2]One way analysis of arcsine transformation of the per cent values for hatchability and % females revealed no significant differences (P .05)

[3]One way analysis of body weights of males and females showed no significant differences among treatments (P .05) at time of hatch, four weeks or eight weeks of age

Table 4

The effect of ultrasound treatment through the shell on 1 or 3 day old chicken embryos with respect to hatchability, sex ratio and mean body weight using a 2.25 MHz. or 5 MHz. transducer at <0.94 mW/cm.^{2} and 0.94 mW/cm^2, respectively, for 3 minutes in the given couplant.

Treatment[1]

Day of Incub. before treat.	Freq-uency in MHz.	Media[2]	Hatchability[3] Rep. 1	Hatchability[3] Rep. 2	% Females[3] Rep. 1	% Females[3] Rep. 2	Mean Body Weights[4] Hatch Male	Hatch Female	4 weeks Male	4 weeks Female
0	0	-	88	100	75	44	46.4	45.8	579	519
1	2.25	water	90	80	33	62	46.8	43.5	585	529
1	2.25	S.C.	75	77	83	71	45.3	45.8	577	554
1	5	water	88	80	75	50	46.8	46.3	637	557
3	2.25	water	90	88	66	37	45.8	45.7	621	551
3	2.25	S.C.	80	80	50	42	45.2	46.1	552	535
3	5	water	100	87	40	57	45.8	48.5	602	548

[1]Each treatment started with two replicates of ten eggs each

[2]S.C. stands for sour cream

[3]No significant differences between treatments at P .05 level as determined by one way analysis of arcsine transformation of % figures

[4]No significant differences between treatments of P .05 level when males and females analysed independently

Table 5

The effect of ultrasound treatment through the shell on 1, 2 or 3 day old chicken embryos with respect to hatchability, sex ratio and mean body weight using a 2.25 or 5.0 MHz. transducer at <0.94 mW/cm.^{2} and 0.94 mW/cm.^{2}, respectively, for 3 minutes in sour cream medium

Treatment[1]									
Day of Incu. before treat.	Frequency in MHz.	Hatchability[2] Rep. 1	Hatchability[2] Rep. 2	% females[3] Rep. 1	% females[3] Rep. 2	Mean body weights[4] Hatch Male	Hatch Female	4 week Male	4 week Female
Control	0	60	73	55	63	37.5	36.1	433	391
1	2.25	66	40	40	50	37.2	36.4	413	442
2	5	40	60	20	55	36.6	35.8	399	399
3	2.25	53	86	50	46	37.8	36.3	426	401
3	5	40	66	33	80	36.8	37.1	442	422

[1]Each treatment started with 2 replicates of 15 eggs

[2]Hatchability was calculated as a per cent of eggs set since determination of exact number of infertile eggs was impossible due to the heavy bacterial infection

[3]No significant differences between treatments of P .05 level as determined by a one way analysis of arcsine values of % figures

[4]No significant differences between treatments at P .05 level where males and females analysed separately

Table 6

The effect of ultrasound treatment through the shell on 1 day old chicken embryos with respect to hatchability, sex ratio and mean body weight using a 2.25 MHz. transducer at 1.51 mW/cm.^{2} or 28.6 mW/cm.^{2} for 3 minutes, using a special gel couplant.

Treatment[1]	Hatchability[2]		% females				Mean Body Weights[3]			
	Rep. 1	Rep. 2	Rep. 1	Rep. 2	Hatch Males	Females	4 week Males	Females	5 week Males	Females
Stationary control	92	100	46	53	38.5	36.8	581	517	799	704
Travelling control	93	100	64	35	39.0	36.8	572	518	794	706
Low amplitude 1.51 mW/cm.^{2}	100	85	35	33	37.7	36.1	571	510	789	692
High amplitude 28.6 mW/cm.^{2}	100	92	60	69	38.3	35.8	578	501	812	688

[1]Each treatment started with 2 replicates of 15 eggs

[2]No significant differences between treatments (P .05) as determined by one way analysis of the arcsine transformation of % figures

[3]No significant differences between treatments (P .05) when males and females are analysed separately

Table 7

Basic description of ultrasonic equipment used and the experiment in which they were employed.

Diagnostic Machine	Frequencies of Transducer and Type	Amplitude Setting of Generator	Radiation Pressure	Intensity at Transducer Face	Cavitation	Experiments in which equip. was employed listed by number
Sono Medic Model 200 U	2.25 MHz. Non-focused	Highest	<2.69mW	<0.94 mW/cm.^{2}	none	1,2,3,4
		Lowest	<2.69mW	<0.94 mW/cm.^{2}		1
	5 MHz. non-focused	Highest	2.7mW	0.94 mW/cm.^{2}	none	2,3,4
Hoffrel Sonoscope 101	2.25 MHz. non-focused	High (1600 volt pulse	80.6mW	28.6 mW/cm.^{2}	none	5
		Low (400 volt pulse	4.3mW	1.51mW.cm.^{2}	none	5

Table 8

Some ultrasonic properties of various egg components as measured at various frequencies.

Material	Density (P) $gm/cm.^3$	Velocity (V) $X10^5$ cm/sec.	Impedance $X10^5$ (=Pr)	Attenuation dB/cm.	Frequency
Albumin	1.04	1.529	1.59	--	5.3 MHz.
Egg Yolk	1.03	1.516	1.56	5.0 ± 1.0[1]	5.3 MHz.
Egg Shell	2.18	N.A.	N.A.	40 dB[2]	2 MHz.

[1]This represents only the absorption coefficient portion of the loss in attenuation.

[2]This measurement was calculated directly.

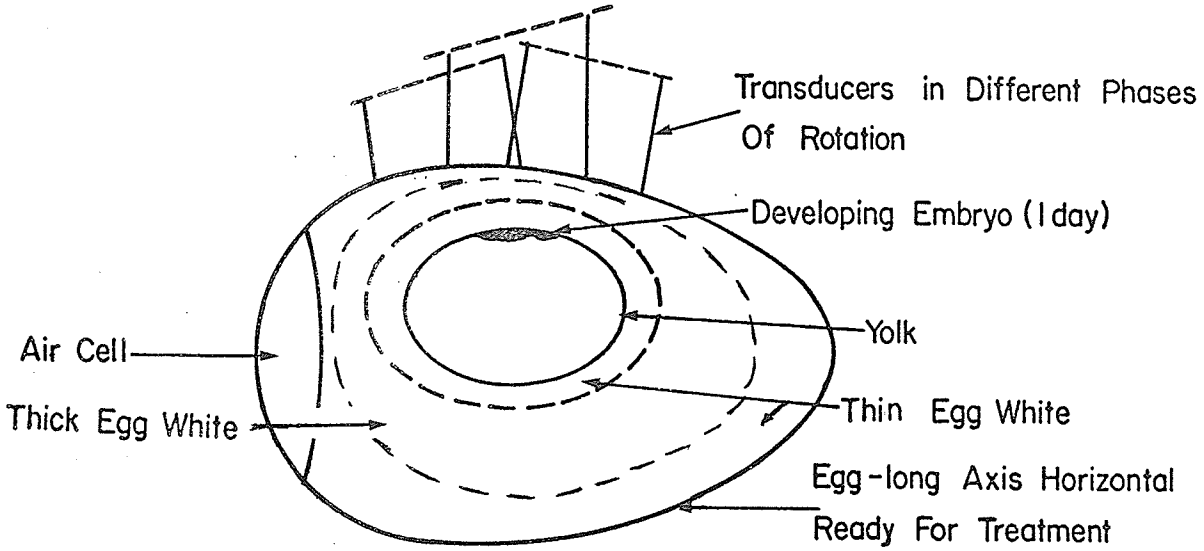

Figure 1. Position of the ultrasonic transducer face held in contact with the shell relative to the embryo during three of the five exposure positions.

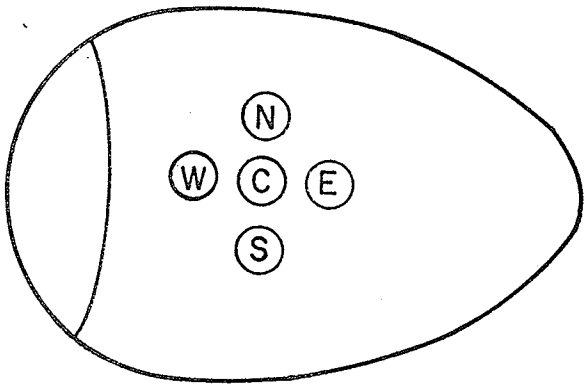

Figure 2. Top view of egg showing ultrasonic exposure pattern. The transducer was held at position C for 1/2 of exposure time and at N,S,E, & W for 1/8 of exposure time respectively.

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APPENDIX 1

GLOSSARY

Acoustic absorption coefficient: For a given material the number of decibels by which the material reduces the intensity of ultrasound per centimetre of travel through it.

Acoustic coupling: The ability to pass acoustic energy from one material to another.

Acoustic load: A material or object coupled to a transducer in such a way that it receives ultrasonic power from it.

Attenuation: A reduction in power level. The opposite of amplification. Measured in decibels.

Capacitance: The ability to store an electrical charge.

Continuous waves: Originally applied to radio transmission to describe that form in which the transmitter gives out radio frequency power continuously. Now applied to the type of irradiation in which the ultrasonic power is delivered without any interruption.

Coupling coefficient: The proportion of the acoustic energy passed from a crystal to a perfectly matched load.

Crystal: Originally a material in which the atoms were arranged in a perfectly uniform pattern. The term is now applied also to the ceramic materials though these are not strictly speaking crystals.

Curie effect, also Piezo-electric effect: The observation by P. & J. Curie that certain crystals develop an electric charge when a mechanical pressure or tension is applied. The reverse piezo-electric effect is the production of mechanical strain in a crystal by applying electrical charge.

Cycle: Here usually applied to one complete repetition of a recurring phenomenon.

Damping: A tendency of oscillations to lose their power. This applies whether the oscillation is electrical or mechanical.

Decibel: The unit of amplification and attenuation. 1/10th of a Bel. One decibel is an increase in power of 26 per cent. or a reduction of 20 per cent.

Degassed: Deprived of its dissolved gas, usually by boiling with a layer of impermeable liquid above, such as liquid paraffin.

Echo-sounding: The use of ultrasonic pulses to measure the depth of water under a ship.

Fraunhofer zone: The zone of radiation beyond the furthest null point.

Frequency: The rate at which a recurring phenomenon recurs. Measured usually in cycles, kilocycles or megacycles.

Frequency constant: For a piezo-electric material the thickness in cm. multiplied by the natural resonant frequency in cycles per second gives a constant, the frequency constant, which is half the velocity of sound in the material in cm. per second.

Fresnel zone: The zone near a transducer in which diffraction produces a number of null points.

Fundamental: In a mixture of mathematically related frequencies, usually the frequency present which has the highest amplitude, those frequencies which are multiples of the fundamental being harmonics and those frequencies that are fractions of the fundamental being sub-harmonics or partials.

Gas cavitation: The liberation of dissolved gas from a liquid by ultrasound to produce small cavities filled with gas. These form bubbles by coalescing.

Generator: Strictly anything that produces. In physics this wide meaning often applies.

In engineering applied usually to a device that generates electrical power such as a dynamo for d.c., an alternator for a.c. and a radio-frequency oscillator device for r.f. power. The last meaning is that most common in ultrasound.

Harmonic: A frequency that is a fairly small multiple of the fundamental

Hertz: 1 cycle per second (Hz)

Impulse: Usually here applied to the short ultrasonic wavetrain.

Interface: The plane where a material having one specific acoustic impedance is continuous with any material having a different specific acoustic impedance.

Interference: Here usually the phenomenon in which two or more radiations of similar frequency appear to be added or subtracted.

Kilohertz: 1,000 cycles per second. Abbreviated correctly to KHz, (Kilocycle)

Longitudinal wave: A vibrational wave in which the wave is propagated in the same direction as the particle moved backwards and forwards.

Matching: This can be either electrical or acoustic. In each case it is the selection of operating conditions to give good transfer of power.

Megahertz: One million cycles (usually per second is understood). Abbreviated to MHz. (Megacycle)

Oscillation: Rhythmic variations in the value of some quantity whether electrical or mechanical.

Oscilloscope: Strictly any device which permits waves to be seen. Hence nearly always in the form of a cathode ray tube.

Radiation pressure: The steady pressure exerted on any material that absorbs or reflects ultrasound, pushing it away from the source of the ultrasound.

Refraction: The angulation when sound or ultrasound passes from one medium into another of different velocity.

Resonance: See Chapter 7. The frequency at which oscillations are most easily produced.

Side-lobe: A beam of ultrasound emerging at an angle to the axis of a transducer and separated from the main lobe by a null point.

Sonar: The use of ultrasound by ships to detect objects whether ships, whales, fish or the ocean bottom.

Standing waves: An interference phenomenon usually caused by reflection where activity in some places appears to be very high and very low in others, as if the waves were standing still.

Streaming: The local movement of a liquid as the result of ultrasonic energy being absorbed by some of the liquid but not by the rest.

Surface wave: Vibrational waves in which vibrations are confined to the surface layer of a solid material.

Transducer: Any device which converts electrical energy into mechanical energy or vice versa.

Transformer: Literally anything that transforms. Usually applied to a device composed of two windings at least, though some turns may be in common. At mains or audio frequency the windings pass round laminated iron cores. At radio-frequencies the core may be of ferrite or air. In acoustics the term has a much wider meaning approximating to the literal.

Ultrasonic: Occurring at a frequency higher than can be detected by the human ear.

Vapour cavitation: Cavitation produced by ultrasound in a liquid with no dissolved gas. The cavities therefore contain the vaporized liquid only. This requires a higher intensity than does gas cavitation.

Volt: The unit of potential. Strictly 10^8 electro-magnetic units of potential difference or 1/300 of an electrostatic unit, these being defined in terms of ergs and dynes.

Watt: The unit of power. The product of the amperes and the volts gives the number of watts in d.c. conditions. In a.c. however this only applies with a resistance unassociated with inductance or capacitance. 746 watts = 1 horsepower.

Wave front: When an acoustic wave is being propagated an imaginary surface passing through all those particles which are in the same phase of vibration, constitutes a wave front.

Wavelength: The distance between a point in an oscillation and the corresponding point in the next.

Wavetrain: The group of oscillations between one period of inactivity and the next.

EFFECTS OF LOW TEMPERATURE STRESS DURING INCUBATION ON EMBRYONIC DEVELOPMENT AND POST-HATCH GROWTH RATE

Introduction

Artificial incubation of avian eggs has been practiced by man since the time of the Ancient Chinese and Egyptians (9). It was based largely on a trial and error method of reaching and maintaining a suitable temperature for development and depended greatly on the experience of the individual in charge. According to Landauer (9), the first incubators to use thermometers or thermostat devices appeared in the 15th and 16th centuries, but is has not been until the 19th and 20th centuries that large scale artificial incubation techniques have flourished in the northern hemispheres.

With increasing importance of artificial incubation optimum conditions for a successful hatch were established. For incubation of chicken eggs the following temperatures have been recommended: between 38.9°-39.4°C. (102-103°F) for still air incubators, and between 37.2°-37.5°C. (99-100°F.) for forced air ventilated incubators (9).

Some controversy exists as to whether the temperature should remain constant throughout the incubation or whether cooling of eggs during incubation may produce beneficial effects on either hatchability or post-hatching growth of chicks (1, 8, 9). In nature, the exposure of embryos to temperatures below optimum for incubation occurs when a brooding bird is forced from the nest by a predator or leaves the nest to obtain food. Even in the late stages of incubation, grouse have been reported to spend as long as 2 hours away from the nest (5). Thermal stress during incubation has been suggested (1, 13) as a means of

eliminating the weaker chicks before hatch, thus leaving the stronger, faster growing chicks to hatch. This could lead to a possible use of temperature stress as a selection aid in genetics if positive beneficial results, i.e. improved viability or post-hatch growth, could be established.

LITERATURE REVIEW

Embryonic Thermal Regulation

The chick embryo functions initially as a poikilothermic animal (20, 21, 24). However, the question as to when the chick becomes a homeotherm has caused some debate. Romanoff (20) originally concluded that the chick became homeothermic after ten days of incubation. He based his conclusion on the fact that after ten days of incubation, a temperature stress of 29°C. for twenty-four hours had little effect on development whereas before the tenth day this temperature greatly delayed the development and time of hatch. He (21) later modified his view proposing that the embryo underwent a more gradual transition and did not become fully homeothermic until the 5th day after hatching. This has been substantiated by Romijn and Lokhorst (24) who have shown that the embryo behaves as a poikilotherm until hatch time. By the time the chick has hatched, it has shifted to a predominantly homeothermic state, which becomes stabilized by 5-7 days after hatch. In their studies (24) they measured the heat production and internal temperature change of the embryo in response to changes in environmental temperatures. They (24) reported that sometime between the 10th and 13th days of incubation the embryo began to maintain a body temperature 1 to 2°C. higher than the surrounding environment, indicating the beginnings of homeothermy. However, for an embryo which had pipped but not hatched, the heat production and the internal temperature decreased with a lowering of the externa temperature a poikilothermic reaction. In the

newly hatched chick as the external temperature dropped, the internal temperature fell also but the heat production began to rise, indicating a homeothermic reaction. By 5-7 days after hatch this reaction was well enough established to fully maintain a constant internal body temperature. Further evidence of a major change occurring at hatch time was provided by Moreng and Shaffner (11). Using lethal temperatures, they (11) found that up to hatch time the internal lethal temperature was (-2 to -1°C) (29-30°F.), whereas at one day of age the lethal temperature was 15.5°C. (60°F.).

Effect of Cold Temperature Stress during Incubation

Reviews of literature from the late 1800's and early 1900's by Landauer (9), Romanoff (20), and Taylor et al. (28) indicated that the general effect of low temperature stress during incubation was retardation of embryonic development and increased embryonic mortality. The advent of electrical incubators led Taylor et al. (28) to investigate the effect of short periods of cooling such as may occur in a power failure. In 1933 they (28) reported on the effect of shutting off the incubators for 12 hour periods and allowing them to cool to a room temperature of 21°C. (70°F.). Embryos were treated at every stage of development from 0 to 20 days of incubation. The increased incubation time required was 18-20 hours and the hatchability averaged 3.4% lower than the controls. Embryos chilled during the second and third week of incubation showed a higher mortality than those chilled during the first week. The increase in mortality did not occur during or immediately following cooling but during the normal peak mortality periods of 0-8 days and 17-21 days of incubation (9, 22). Studies by Romanoff (20) using temperature exposure of 29°C. (84.2°F.) for twenty-four hours at

0, 4, 8, 12 and 16 days of incubation had a similar retardation of growth and delay in hatch time. Hatching time was delayed longest for eggs treated during the early stages of incubation while embryonic mortality increased with embryo age at time of treatment.

A considerable amount of work has been done by Moreng and his co-workers (11, 12, 13, 14, 15) on cold stressing and lethal embryonic temperatures. In 1951 they (11) conducted a series of temperature-time combinations on embryos from 1 to 20 days of incubation. On removal from the incubator, groups of eggs were subjected to -23.2°C. (-10°F.) for varying lengths of time. The internal temperature was recorded by use of a wire thermocouple inserted in the egg. Eggs exposed in this manner usually reached an internal temperature of -1.6°C. (29°F.) in 55 minutes. This temperature was maintained for approximately the next 2 hours until the egg was completely frozen. The temperature then declined until it reached -23.2°C.The total time in the cooler required to cause 50% mortality was considered the lethal exposure time and the internal temperature at this time was the lethal temperature. In this study (11) the internal lethal temperature was always -1 to -2°C. (30 to 29°F.). The lethal exposure times were: 95 minutes for embryos 1 to 6 days of age; 70-75 minutes for those 7 to 14 days of age and 80-85 minutes for those embryos 15-20 days of age. In later studies (12), using the same methods, the embryos showed the greatest resistance to a cold stress during the first 3 days of incubation, and the greatest susceptibility during the 18th, 19th and 20th days of incubation. This was determined by increasing the exposure time by 5 minutes over the lethal times determined in previous experiments (11) and noting which periods showed the greatest increase in embryonic mortality. Their findings (12) agree

with those of other workers (9, 20, 28). In embryos treated for extended periods at -23.2°C. (-10°F.) the major cause of death was due to the formation of ice crystals within the body cells (14). Moreng et al. also conducted some studies on cooling which did not involve freezing of embryos (14, 15). At an internal temperature of 0°C. embryos could tolerate much longer periods of exposure at any given age as compared to -1.66°C. (29°F.) but mortality still followed the same pattern of increasing with age (14). It was also concluded that the more rapidly the embryo was cooled to the desired constant temperature, the more detrimental was the temperature stress even though the total length of exposure at the constant temperature was equal (14). Embryos exposed to a temperature stress of 12.7°C. (55°F.) survived up to 19 hours exposure up to 11 days of incubation (15). However, after the fourth day of incubation hatchability began to drop as the age of the embryo receiving treatment increased. After eleven days of incubation the embryos died shortly after or during the nineteen hour exposure at 12.7°C. (55°F.). In general they (13, 14) found that hatchability of the embryos which survived the temperature treatment decreased as the age of embryos at the time of treatment increased. In one trial, Moreng et al. (13) raised the chicks which survived the treatment of -10°F. for the lethal time limits. They (13) found that the treated chicke were heavier at one day of age but grew slower than the controls and had a higher mortality to six weeks of age.

Morgan and Tucker (16) attempted to alter the sex ratio of chicks by temperature stress imposed on the 7th, 8th, 9th or 10th day of incubation. On the above days of incubation, fertile eggs were cooled to 6°, 21°, or 35°C. for 3 hours. Although no differences were found in

the sex ratio, the 6°C. and 21°C. treatments had a slight improvement in hatchability over the controls. Kosin (8) has suggested in a recent review of literature that periodic lowering of the temperature of the incubator and hence, of embryos, may be beneficial in increasing hatchability, especially for water fowl.

The effect of cold stress during incubation on post-hatch growth rates has been studied recently by Buckland (1, 2, 4). He (1, 2) exposed embryos on the 17th day of incubation to 21.2°C. (70°F.), 11.3°C. (52°F.) or 5.2°C. (41°F.) for 4, 8, 12, 16, 20, or 24 hour periods. In regards to hatchability, there was no significant difference between the controls and any treatment done at 21.2°C. However, for exposures at 11.3°C. and 5.2°C. a drop in hatchability occurred after 12-16 hours and after 12 hours respectively. After 24 hours exposure at 11.3°C. hatchability was down to 1-5% while in the 5.2°C. group it was 0%. Hatch weight increased with length of exposure at all cold stress treatments except for those treated at 21.2°C. Prolonged exposure at 11°C. and exposure of 12 hours or more at 5°C. decreased body weight at 2, 4 and 6 weeks of age and increased the mortality. However, the treatment at 5.2°C. for 4 and 8 hours at 17 days of incubation produced a significant increase in body weight of males at 4 and 6 weeks. Females showed a tendency toward the same results. These results were found for two consecutive years (1, 2). However when two different light regimes were introduced into the rearing environment; intermittent versus continuous, the growth response was only evident for chicks from the 5.2°C. treatment which were grown in the continuous light environment (4).

It appears that most of the work which has been done on low

temperature stress during incubation has been concerned with embryo growth and hatchability. It has been shown that low temperature stress slows the development of the chick and extends the required incubation time (9, 11, 20, 28). The embryo has proved most resistant to cold stress during the first week of incubation and least resistant during the 3 days before hatching (9, 11, 12, 20, 28). The greatest delay in hatch rate and the most deformities occur when cold stress is applied during the first week. Faster rates of cooling of embryos resulted in high immediate mortality and lower overall hatchability than comparative treatments chilled at a slower rate (14). For embryos cooled below freezing point, -1 to -2°C., the formation of ice crystals in the tissue has been the main cause of death (14). The exact cause of death in embryos not frozen has not been determined and has been merely attributed to physiological shock (15). In comparing the various studies using different temperatures (9, 14, 15, 28), it can be seen that embryonic mortality rises with increasing exposure time and decreasing temperature, indicating that death is not solely due to interruption of incubation. In regards to the effect of cold temperature stress during incubation on post-hatching performance, only a few reports are available (2, 1, 4, 13). Most temperature treatments have been detrimental to growth and resulted in increased mortality (1, 2, 4, 13). However one temperature-time combination of 5.2°C. for 8 hours at 17th day of incubation has been reported to significantly increase the growth of male chicks (1, 2). It has been suggested (1, 2) that the response was due to elimination of weaker embryos which would have produced less vigorous chicks.

Due to the relative lack of information on the post-hatch

performance of low temperature stressed embryos and unusual patterns of certain studies, a further study into this field was initiated.

METHODS

General Materials and Methods

Except for the preliminary study, Experiment 1, all experiments were conducted using the same basic methods and equipment. The variables studied were the temperature of exposure, the length of exposure and the number of days of incubation before exposure. Experiment 1 included at least two levels of each variable, whereas the remaining experiments tested only one temperature per trial, usually at only one stage of incubation, but for varying lengths of exposure.

The eggs in Experiment 1 were obtained from a commerical broiler breeder flock. In all other experiments, eggs of an egg type commercial W. Leghorn hybrid stock, mated at the University of Manitoba, were used. Thus the chick weights from Experiment 1 were considerably higher than those in the remaining trials. All eggs were incubated, before and after treatment, in a Robbins Model 11H incubator and hatcher at 37.5°C. (99.5°F.). Eggs were transferred to the hatcher section at 18 days of incubation. All eggs were subjected to 22 days of incubation to allow for delays in development and time of hatch caused by cooling treatments.

A thermostatically controlled walk-in cooler 5' wide x 10.5' long x 7' high was used for all temperature treatments. Two twelve-inch fans forced air over a 24" x 14" x 3" cooling-heating grid, to provide air circulation in the cooler. The eggs were placed large end up (long axis vertical) in fibre trays with a maximum of 15 eggs in a thirty egg tray to allow for uniform cooling of the eggs. The trays were set on a platform

2.5 feet above the floor.

A multi-point temperature recorder was employed, using from 4 to 8 thermocouples, to record the temperature of the cooler and the internal temperature of the eggs during the treatment periods for all tests except Experiment 1. A thermometer placed in water, which had been allowed to reach the ambient temperature in the cooler, was used to determine and set the temperature of the cooler since the air thermometer fluctuated with slight short changes caused by opening and closing of the cooler door. During treatment a thermocouple was immersed in the water to provide a continuous record of the room temperature. In Experiments 4 and 5 an open water bath was used but was replaced by a closed 600 ml Erlenmyer flask since the thermocouples in the open water bath gave a reading 0.5-1°C. lower than the temperature of the water as measured by the thermometer. This did not occur when the flask was used. Thermocouples were inserted into at least 3 eggs during all treatment periods for all experiments and the change in temperature of the eggs recorded. This was done by making a small hole in the large end of the egg, breaking the membrane and inserting a thermocouple about 1 inch into the egg. For eggs treated after 17 days of incubation, care was taken to not break the larger extra-embryonic blood vessels and the thermocouple was inserted between the head and the breast of the developing chick. The hole was sealed and the thermocouple held in place with masking tape.

All eggs which failed to hatch were broken open and examined to determine fertility. Dead embryos were examined to determine age of death and any morphological abnormalities observed were described. Hatchability was recorded, as per cent of fertile eggs, for all replicates.

After hatching, all chicks were sexed and weighed individually at weekly intervals from 0 to 8 weeks. Mortality records were kept and dead birds examined at the Veterinary Laboratory. All birds were reared in multi-level wire brooding batteries to 8 weeks of age, except the broiler chicks in Experiment 1 which were moved to floor pens at 4 weeks of age. All birds were raised in a continuous light environment. The same commercial chick starter ration was fed to all chicks in each experiment.

Data were subjected to analysis of variance (25) and where appropriate, means were tested by a Duncan's Multiple Range Test. All per cent data were converted to arcsine values for analysis. Data from all replicates per treatments were pooled by sex for analysis of variance of mean body weights. An Olivetti Underwood Programmer 101 desk top computer was used to conduct this analysis.

Experiment 1

This preliminary experiment studied the effects of various cold temperature stresses during the early and late stages of incubation on embryonic development and on post-hatching performance. Four hundred and thirty-two commercial broiler eggs were divided into four groups containing four replicates of twenty-seven eggs. The following treatments were used:

A) Control.

B) 24 hours of exposure to 7.2°C. (45°F.) commencing at four days (96 hours) of incubation.

C) 4 hours of exposure to 3.8°C. (39°F.) commencing at 17 days (408 hours) incubation.

D) 8 hours of exposure to 3.8°C. (39°F.) commencing at 17 days (408 hours) of incubation.

The eggs which were chilled for 24 hours at 7.2°C. were set a day prior to the controls so that they would hatch at the same time as the controls. Those treated at 17 days of incubation (3.8°C.) were set six and four hours ahead of the controls for the 8 and 4 hour exposures, respectively. All eggs received 22 days of incubation to allow for adequate recovery from chilling.

For this experiment, the eggs remained in the incubator trays during temperature treatments rather than as described in the general method. For the treatment which was to be done at 17 days of incubation the cooler had been set at 5°C. (41°F.) to test the growth response reported by Buckland (3, 5). However, during the early part of the treatment the temperature dropped and averaged closer to 3.8°C. (39°F.). The temperature was monitored by periodic visual checks as the multi-point temperature recorder was unavailable for this experiment. Growth studies of the chicks from the group treated at 17 days of incubation were terminated at four weeks of age. The controls and those subjected to 7.2°C. on the fourth day of incubation were moved from brooder batteries to floor pens and continued until 8 weeks of age.

Experiment 2

This experiment was conducted to further study the effect of exposure of embryos at 4 days of incubation to 7.2°C. for 24 hours on post-hatching growth rate.

One hundred and sixty eggs were divided into two groups containing four replicates of twenty eggs. The treatments assigned were:

a) control - no exposure

b) 24 hours exposure to 7.2°C. $\pm$ 1 (45°F.) commencing at 4 days of incubation.

Experiment 3

This experiment was conducted to determine if the limb deformities observed in Experiment 2 were related to a particular stage of incubation and could be affected by the length of exposure or the position of the egg during treatment.

Four hundred and fifty eggs were divided into ten treatments containing three replicates of fifteen eggs each. The treatment consisted of subjecting eggs to 7.2°C. $\pm$ 1 for the following times of exposure and stages of incubation:

1) Control - normal incubation, no exposure - set 1 day later than eggs to be treated.
2) 24 hours exposure at 94 hours of incubation
3) 25 " " " 94 " " "
4) 23 " " " 96 " " "
5) 24 " " " 96 " " "
6) 25 " " " 96 " " "
7) 23 " " " 98 " " "
8) 24 " " " 98 " " "
9) 25 " " " 98 " " "
10) 24* " " " 96 " " "

*the eggs were set so that the long axis of the egg was horizontal rather than in the normal vertical position.

Experiment 4

This experiment was done to further investigate the abnormal development of the pipping muscle observed in embryos, subjected to 3.8°C. for 4 or 8 hours at 17 days of incubation in Experiment I.

One hundred and eighty eggs were divided into three groups containing four replicates of fifteen eggs each. The following treatments were assigned:

1) Control - normal incubation
2) 4 hour exposure of embryos to 3.8°C. $\pm$ 1 (39°F.) commencing at 17 days of incubation
3) 8 hour exposure of embryos to 3.8°C. $\pm$ 1 (39°F.) commencing at 17 days of incubation.

All eggs containing thermocouples were broken open and the embryos examined for viability immediately following the temperature treatments.

Experiment 5

In this experiment 5 and 6 hours of exposure were studied to see if the threshold value for zero hatchability as obtained in Experiment 4 lay between these time limits for embryos exposed to 3.8°C. at 17 days of incubation.

Two hundred and forty eggs were divided into three treatments containing four replicates of twenty eggs. The following treatments were assigned:

1) Control - normal incubation
2) 5 hours of exposure of embryos to 3.8°C. $\pm$ 1 commencing at 17 days of incubation
3) 6 hours of exposure of embryos to 3.8°C. $\pm$ 1 commencing at 17 days of incubation.

Experiment 6

This experiment was designed to study the comparative morphological

development of the hatching muscle as affected by the following temperature-time treatments as well as to continue to study post-hatch growth response.

Three hundred and twenty eggs were divided into four treatments containing four replicates of twenty eggs treated in the following manner:

1) Control - normal incubation
2) 4 hours exposure to 3.8°C. $\pm$ 1 commencing at 17 days incubation
3) 5 hours exposure to 3.8°C. $\pm$ 1 commencing at 17 days of incubation
4) 8 hours exposure to 3.8°C. $\pm$ 1 commencing at 7 days of incubation

One egg was removed from each replicate of each treatment every twelve hours after the 18th day of incubation until 21.5 days of incubation and the embryos examined. The wet weight of the embryo was recorded. The hatching muscle, M. complexus, was examined for presence and degree of edema and hemorrhage. The muscle was then removed, a wet weight recorded, and then placed in a 10% formaldehyde solution. Paraffin sections were prepared and stained routinely with hematoxylin and eosin for histological comparison. The weight of the hatching muscle was converted to a per cent of body weight for comparative purposes. Eggs which were not removed were allowed to hatch and post-hatch chick growth was studied.

Experiment 7

Since Buckland (1, 2) reported a growth response in post-hatch body weight for embryos exposed to 5°C. for 8 hours at 17 days of incubation, this temperature was studied in this trial.

One hundred and eighty eggs were divided into three treatments containing four replicates of fifteen eggs. Treatments were assigned as follows:

1) Control - normal incubation
2) 4 hours exposure to 5°C. $\pm$ 1 (41°F.) commencing at 17 days of incubation
3) 8 hours exposure to 5°C. $\pm$ 1 (41°F.) commencing at 17 days of incubation.

Experiment 8

This experiment was identical to Experiment 7 except for larger replicate size. Both were run concurrently, with Experiment 8 starting several days later than Experiment 7. Both experiments tested the effect of 5°C. on post-hatching growth rate.

Two hundred and forty eggs were divided into three treatments containing four replicates of twenty eggs treated in the following manner:

1) Controls - normal incubation
2) 4 hours exposure at 5°C. $\pm$ 1 commencing at 17 days of incubation
3) 8 hours of exposure at 5°C. $\pm$ 1 commencing at 17 days of incubation.

The relative humidity of the cooler was measured during treatment by means of wet and dry bulb thermometers.

Experiment 9

Exposure to 5°C. was made at two different stages of incubation in this experiment. It was learned that Buckland's (3) exposures

started about 6 hours earlier than those in Experiments 7 and 8. A comparative study using exposure to 5°C. at 16 days + 21 hours of incubation (Buckland's time - 3) versus 17 days + 3 hours was undertaken to see if this difference in stage of development during treatment would explain the difference in hatchability or post-hatch performance between Buckland's (1, 2) work and Experiments 7 and 8.

Three hundred eggs were divided into five treatments containing three replicates of fiteen eggs each. The treatments were assigned as follows:

1) Control - normal incubation
2) 4 hours exposure at 5°C. $\pm$ 1 commencing at 16 days + 21 hours of incubation
3) 8 hours exposure at 5°C. $\pm$ 1 commencing at 16 days + 21 hours of incubation
4) 4 hours exposure at 5°C. $\pm$ 1 at 17 days + 3 hours of incubation
5) 8 hours exposure at 5°C. $\pm$ 1 commencing at 17 days + 3 hours of incubation

Relative humidity of cooler during treatment was recorded. Eggs with thermocouples were checked after treatment for viability. Several groups were incubated for 23 days to see if they would hatch if given more time.

RESULTS

Experiment 1

For ease of discussion the treatments will be coded as follows:

A) Control

B) 4 day - 24 hour - meaning those exposed to 7.2°C. $\pm$ 1 for 24 hours at 4 days of incubation

C) 17 day - 4 hour - meaning those exposed to 3.8°C. $\pm$ 1 for 4 hours at 17 days of incubation

D) 17 day - 8 hour - meaning those exposed to 3.8°C. $\pm$ 1 for 8 hours at 17 days of incubation.

Hatchability percentages are presented in Table 1. The 17 day - 8 hour treatment was significantly (P .05) lower than all other treatments, while both the 17 day - 4 hour and 4 day - 24 hour treatments were non-significantly lower than the controls.

Table 1 presents a breakdown of the number of embryonic deaths which occurred during three periods of incubation. Under normal conditions the period from 0-8 days of incubation and that after 17 days of incubation (17+) are the peak periods of mortality and are nearly equal (9, 22). In the first period, 0-8 days, the 4 day - 24 hour treatment contained over 50% of the mortality. Probably this was largely due to the imposition of the temperature treatment within this period as examination of the embryos revealed that death occurred at 4 to 5 days of incubation in the majority of embryos. The 17+ period, which included those embryos which failed to hatch by 22 days of incubation also exhibited high mortality. Over 75% of the mortality in this,

occurred in the 17 day - 8 hour treatment. Figure 1 illustrates the arrangement of the replicates for the 17 day - 8 hour treatment in the incubation trays as they were placed in the cooler. Each rectangle in Figure 1 represents one replicate of 27 eggs denoted by the letters E, F, G, or H. The per cent hatch of fertile eggs and the number of dead embryos in the 17 + day period are also given. It can be seen this low temperature treatment was completely fatal to the two outside replicates, whereas a limited number survived from the inner replicates, probably from the central area. This pattern suggested that the cooling took place from the outside to the center of the treatment, i.e. the outer eggs acted as insulation and heat source for the inner eggs. It was felt that if the eggs had been separated and every egg allowed to cool at the same rate, hatchability would likely have been zero for all replicates. In all other experiments the eggs were separated to allow uniform chilling of eggs.

The dead embryos in the 17 day treatments had two striking morphologic characteristics which are summarized in Table 1. Fifty per cent of the embryos examined were not in the usual normal position for a chick which is about to hatch (9, 23). The normal orientation for the chick prior to and during hatch is with the head at the large end of the egg, with the beak usually pointing to or in the air cell. In many of these embryos the head was found at the small end of the egg or part way down the side of the egg pointed down to the bottom. The second feature was the very noticeable swelling of the hatching muscle (M. complexus), giving the chick a hydrocephalic appearance (Figures 2, 3, and 4 Muscle complexus is a paired muscle overlying Muscle spinalis and Muscle biventes cervicis and originates from the neural spines of cervical

vertebrae 3, 4, 5 and from fascia overlying deeper muscles. It inserts on the posterior edge of the parietal bones and provides the power for rupture of the shell at hatching (18). Cutting open the skin over the muscle revealed a greatly enlarged muscle, larger than any examined from controls or 4 day - 24 hour treated. A large amount of jelly-like fluid (edema) was present surrounding and throughout the muscle, (Figure 3, 4) and hemorrhage varying from slight to extensive was visible 50% of the time (Figure 5).

Mean body weight at hatching and at 4 and 8 weeks are presented in Table 2. Hatch weights for treatments were significantly different ($P < .05$) as shown by Duncan's Test (Table 3). The 4 day - 24 hour treatment resulted in heavier chicks than all other treatments. At 4 weeks the temperature treated groups were non-significantly heavier than the controls with the 4 day - 24 hour treatment being the heaviest. Growth studies of the 17 day treatment were terminated at four weeks. The 4 day - 24 hour treatment was significantly heavier than the controls at 8 weeks ($P < .05$).

Mortality was approximately the same for all treatments from 0-4 weeks. The controls, 4 day - 24 hours, 17 day - 4 hours, and 17 day - 8 hours lost 2, 3, 3 and 0 birds, respectively. Mortality was higher for the 4 day - 24 hour treatment as compared to controls (7 vs. 2) from 4 to 8 weeks. This was due to birds killed because of the occurrence of perosis in the temperature stress groups.

Experiment 2

The temperatures of the cooler and the internal temperatures of the egg during exposure to 7.2°C. for 24 hours at 4 days are listed in Table 4 and shown in Figure 6. The egg reached the approximate temperature

of the cooler within about 3 hours of exposure. The cooler temperature averaged 7.5 °C.

Hatchability was significantly (P .05) lowered as shown in Table 5. The highest embryonic mortality, listed in absolute figures, occurred in the 0-8 day period for the treated embryos. Examination revealed that the majority of the embryos died between the 4th and 5th days of incubation. Three embryos which died after the 8 day period had deformed limbs. Two embryos had short stubs replacing one wing and one had a shortened leg. Thirteen of the chicks which hatched also had deformed limbs. Two involved shortened wings, one had both a wing and a leg affected and ten had deformities of the feet and legs. The wing abnormalities ranged from replacement of the wing by a hard skin covered knob approximately 5 cm. in length to wings about half the size of the normal wing on the opposite side of the body. Leg and feet deformities consisted of severely curled toes, shortened and thickened leg bones with club shape feet, short bony stumps or the complete absence of a leg. (Figure 7) The deformed chicks were weighed, sexed and recorded for each group, then killed and examined for internal abnormalities. None were found.

Mean body weights and post-hatch mortality are presented in Table 6. The males and females were combined for analysis of hatch weight but these were analysed separately at 4 and 8 weeks. No significant differences (P .05) were found for mean body weight although the females from the treated group were 40 g. lighter at 8 weeks.

Experiment 3

In the third experiment using exposure to 7.2°C. for 24 hours at 4 days, the temperature recording machine ceased to function properly

3 hours after treatment began and its use was terminated. The temperatures recorded for this period are found in Table 4 and Figure 6. Visual examination of the thermometer revealed a rise in temperature to 10° C. (50°F.) by the end of the 24 hour treatment, due to a breakdown of the cooler motor. As the temperature recorder was not working, the exact length of time which the cooler was not at 7.2°C. $\pm$ 1 was unknown. However it was probably not more than a couple of hours as the cooler had only risen 2.8°C.

The hatchability means are given in Table 7, which also contains the Duncan's test showing the significant differences between treatments (P .05). The controls had a higher hatchability than all treatments except the eggs treated at 94 hours of incubation for 23 hours exposure and those treated at 96 hours of incubation for 23 hours. Treatment commencing at 96 hours of incubation for 23 hours of exposure was significantly (P .05) different than treatment at 98 hours incubation for 25 hours exposure. There appeared a slight trend indicating that the longer the treatment and the later the time of exposure, the greater was the decrease in hatchability. Summaries of embryonic mortality by periods and of deformed embryos are presented in Table 8. As in the previous experiment, the embryonic mortality was greatest in the 0-8 day period, mainly at the 4th to 5th day of development. Two embryos from the later periods had abnormally formed limbs (one embryo minus a leg and one with a shortened wing and club foot). The number and types of deformed chicks appear unrelated to any one treatment (Table 8). Ten per cent of the chicks which hatched from the treated eggs were malformed whereas none of the chicks from control groups showed any malformation. The deformities were of the same type as described in Experiment 2. The nine birds with the deformed legs were killed since they could not walk

properly. However the birds with only wing deformities remained on test and their weight did not appear affected by the small wing.

Hatch weights were analyzed (males and females combined) and the means are given in Table 9. Significant differences in mean hatch weight, determined by Duncan's test are shown in Table 9 . There were no significant differences (P .05) between treatment for the 4 and 8 week body weight means for either males or females (Table 10). Table 10 also contains post-hatch mortality which did not appear to be affected by treatment. Five birds from treatment for 23 hours at 98 hours of incubation died three days after hatch, due to inadvertent lack of drinking water.

Experiment 4

Table 4 and Figure 8 show the temperature of the cooler and the internal temperature of the egg during treatment at 3.8°C. for 4 and 8 hours commencing at 17 days of incubation. The internal temperature of the egg dropped to almost the recorded level of the cooler by 4 hours, but seemed to remain about a degree above the cooler temperature. Embryos were checked for viability to assure that the insertion of the thermocouple into the egg did not cause death and therefore that the temperature recorded was similar to the temperature to be expected in the eggs containing live embryos with no thermocouple. Eggs which contained thermocouples were broken open and examined immediately after treatment. All embryos were viable as determined by the presence of a slow but steady heart beat.

Hatchability values for the treatments, embryonic mortality and some of the morphology of the dead embryos are presented in Table 11. Exposure to 3.8°C. for eight hours commencing at 17 days of incubation

resulted in zero hatchability after 22 days of incubation. Four hours exposure to 3.8°C. significantly (P .05) lowered the hatchability also, as compared to the controls. The majority of the embryonic deaths occurred after treatment at 17 days. Nearly 50% of those failing to hatch were not in the normal hatching position as described in Experiment 1. About 80% of the hatching muscles of the dead embryos were edematous and 50% were hemorrhagic. According to Rigdon (18) some edema occurs naturally in the area of the hatching muscle during the normal development and hatching of the chick, as well as some hemorrhaging. In this study the presence of edema and hemorrhages has been given a visual scale reading of 1, 2, or 3. Number 1 is interpreted as the lowest value signifying the presence of the condition just slightly greater than normal. For edema value 1 represented up to a 25% increase in fluid and muscle size, value 2 a 25%-75% increase and the value 3 a 75% + increase. In gauging the degree of hemorrhage, value 1 represented from 5 to 20% of muscle covered with hemorrhages, value 2 was 20-40% of muscle area covered and 3 was 40% or more of muscle area covered by hemorrhages (See Figures 4 and 5). The standard for normal was obtained by examination of control embryos and chicks and further substantiated in Experiment 6.

Mean body weights are presented in Table 12 for controls and the four hour treatment. No significant differences were found at any stage of growth between treatments and post-hatch mortality was the same with each group losing 4 birds up to 8 weeks.

Experiment 5

During the 5 and 6 hours exposure of eggs to 3.8°C. $\pm$ 1 commencing at 17 days of incubation, the temperature of the cooler and the internal

temperature of the egg were recorded as listed in Table 4 (Graphed - Figure 8).

Exposure to 3.8°C. for 5 and 6 hours significantly reduced hatchability below the level of the controls (Table 13). For 6 hour exposure the hatchability was 5.6%, so that the threshold time value for zero hatchability probably lies shortly beyond 6 hours of exposure to 3.8°C. using the cooler described in these experiments. The distribution of embryonic mortality was similar to Experiments 1 and 4, occurring mainly after 17 days of incubation. The occurrence of malposition of head, edema and hemorrhage were the same morphological abnormalities as found in Experiments 1 and 4. The chicks were weighed at hatch and then destroyed as the 5 and 6 hour groups had insufficient numbers hatched for a meaningful growth study and statistical analysis.

Experiment 6

In a study of the comparative morphological development of the hatching muscle of embryos as affected by exposure to 3.8°C. $\pm$ 1 for 4, 5 or 8 hours commencing at 17 days of incubation, embryos or chicks from controls and each exposure were examined every twelve hours starting from the 18th day of incubation. A summary of the number of malpositions and the number and degree of edematous and hemorrhagic hatching muscles per treatment from the 18th to 21.5 days of incubation are presented in Table 14 for four examination periods. The number of malpositions was fairly constant increasing slightly by 21.5 days of incubation. No malpositions were found in the control eggs at 18 or 19.5 days of incubation. At 20.5 and 21.5 days of incubation the majority of the control eggs and those stressed for 4 hours at 17 days were hatched.

For these groups the hatching muscles from chicks which had hatched were examined as this represented the condition of the average chick within each group and therefore the normal condition of the muscle per group at these stages. No excess edema and only one incidence of hemorrhage was found in the controls. Neither edema nor hemorrhage had reached abnormal levels for any of the treatments when examined at 18 days of incubation. However, by 21.5 days of incubation almost all the embryos from the 5 and 8 hour treatments had excess edema and hemorrhage of the hatching muscle.

The wet weight of the hatching muscle shown as a per cent of wet body weight for the four treatments at four stages of incubation are given in Table 15A. At 20.5 days of incubation the hatching muscle of embryos which received the 8 hour temperature stress commencing at 17 days of incubation were significantly ($P < .05$) heavier than all other treatments (Table 15B). At 21.5 days, muscle weight from the 8 hour exposure groups were still significantly greater than for the control or 4 hour exposure groups. However the muscle weight of the 5 hour exposure group had increased in weight so that it was no longer significantly lighter than the 8 hour exposure group and was almost significantly heavier than the four hour exposure and the control groups. (Table 15B).

The histological sections were very similar to those described by Rigdon (17, 18). The presence of edema could be seen by an increase in width between the muscle fibres. Increased numbers of red blood cells were found in the tissue between the muscles fibres. The presence of necrotic muscle cells was noted but were very few and much less than the degree reported by Rigdon(18).

The internal temperature of the cooler and of the egg are given

in Table 4 and Figure 8. They are almost identical to Experiments 4 and 5.

Table 16A shows the hatchability, embryonic mortality and morphology of the eggs set for the post-hatch growth study section of Experiment 6.

The eight hour exposure still resulted in zero hatchability. The hatchability for the 5 hour treatment was also significantly (P .05) lower than for the controls or the 4 hour treatment; the latter being insignificantly lower than the controls (Table 16B). Malposition, edema and hemorrhage increased with the length of treatment.

Mean body weights were not significantly different (P .05) at time of hatch, at 4 or 8 weeks of age as shown in Table 17. There was no post-hatch mortality in any group.

Experiment 7

During temperature exposure of embryos to 5°C. $\pm$ 1 for 4 or 8 hours commencing at 17 days of incubation, the internal temperature of the egg and the cooler were recorded as reported in Table 4 and Figure 9. The pattern was similar to those at 3.8°C.

Hatchabilities, embryonic mortalities and the morphological abnormalities are presented in Table 18. Hatchability was zero for 8 hours exposure at 5°C. as it was with 3.8°C. in previous experiments. Hatchability for the 4 hour treatment was significantly (P .05) lower than the controls while the 8 hour exposure was significantly lower than both groups. Embryonic mortality followed the same pattern as that found in the 3.8°C. experiments with the same predominate morphological characteristics.

The mean body weight at hatch, at 4 and 8 weeks of age and the

post-hatch mortality are given in Table 19. No significant differences (P .05) were evident in hatch weight for either sex or for females at 4 or 8 weeks of age. However, the control males were significantly heavier than the treatment males at both 4 and 8 weeks. Mortality was also higher for the treated birds in the first 4 weeks with most deaths occurring during the first few days after hatch.

Experiment 8

During temperature exposure of embryos to 5°C. $\pm$ 1 for 4 or 8 hours commencing at 17 days of incubation, the internal temperature of the egg and the temperature of the cooler were recorded and are presented in Table 4 (Figure 9). The temperature of the egg approximated the cooler temperature after about 4 hours of exposure.

The per cent hatchability was lowered, but not significantly (P .05), by the 4 hour treatment (Table 20). The 8 hour treatment again resulted in zero hatchability. The number of malpositions, edematous and hemorrhagic hatching muscles per treatment are also presented in Table 20.

Mean body weights are given in Table 21. The mean hatch weights for either sex and the mean female body weights at 4 and 8 weeks were not significantly different (P .05). However the 4 and 8 week mean body weights for males were again significantly different (P .05). This time the treatment males were heavier than the control males. Post-hatch mortality was slightly greater for treated chicks than for the controls.

Experiment 9

The eggs were exposed to 5°C. for periods of 0, 4 or 8 hours commencing at either 16 days + 21 hours or 17 days + 3 hours of incubation.

The internal temperature of the eggs and the cooler during treatment are reported in Table 4 and Figure 9. The relative humidity of cooler was 12-17%.

The per cent hatchability and embryonic mortality and morphology are given in Table 22. The 8 hour exposure to 5°C. still significantly reduced hatchability to zero for both treatments. Hatchability for the 4 hours exposure which started three hours after 17 days of incubation was slightly lower than that for the 4 hour exposure at 16 days + 21 hours of incubation, 47% versus 55.5%, respectively, but not significantly. Both were significantly (P .05) lower than the control average of 90%. The number of malpositions and amount of edema and hemorrhage appeared to increase with the advancing stage of incubation during which treatment was started as well as with the length of treatment.

Mean body weights and post-hatch mortality are given per treatment and sex in Table 23. There were no significant differences (P .05) in mean body weight at time of hatch, at 4 weeks or at 8 weeks of age, although the males treated at 17 days + 3 hours and the females treated at 16 days + 21 hours were 30 to 40 g. heavier than controls at 8 weeks. Post-hatch mortality was considerably higher for those hatched from embryos treated to 5°C. after 16 days + 21 hours of incubation.

Some groups were carried for 23 days of incubation. Even after this length of time no chicks hatched from the 8 hour exposure treatments. Nine chicks from different replicates of both 4 hour treatments were assisted by hand out of the shell but only two of these survived. These chicks were not used in computation of results and were counted as embryonic mortality in the 17+ column. Embryos from eggs which had

thermocouple insertion were again checked for viability at the end of treatment. Only 1 out of 8 embryos appeared to have died possibly from loss of blood due to rupture of a major extra-embryonic blood vessel.

DISCUSSION

Experiment 1, testing the effects of cold temperature stress during both late and early stages of incubation at temperatures of 3.8°C. and 7.2°C. respectively, revealed the need of several changes in procedure. The first of these was the separation of the eggs in the cooler to allow uniform cooling of all eggs. The reason for suspecting unequal cooling rates for all eggs was the unusual pattern of mortality as described in the results of the treatment of embryos at 3.8°C. for 8 hours after 17 days incubation (See Figure 1). This suspicion was confirmed by the complete failure of embryos to hatch in this treatment in Experiments 4 and 6 where the eggs were spread out in the cooler as described in General Methods. The second procedure was the addition of the use of a multipoint temperature recorder. The first advantage of this system was that it provided a continuous record of the temperature of the cooler during treatment so that any deviation from the desired treatment, such as occurred in Experiment 1, could be observed and taken into consideration. Unfortunately, the second time a deviation from the desired temperature occurred, in Experiment 3 due to mechanical breakdown of cooler, the recorder had broken down before the cooler failure. Therefore, although the temperature by the end of the treatment had only risen 2.8°C., the length of time was not known and once again emphasized the importance of the temperature recorder. A second major advantage was the use of the recorder to follow the rate of cooling of the internal temperature of the egg during treatments. This provided a better

reference point for treatment comparisons than the temperature of the cooler alone. The rate of cooling and subsequently the length of time the internal temperature of the egg remained at or near the ambient temperature in the cooler could vary among coolers set at the same temperature depending on factors such as air circulation, humidity, or egg position in the cooler. The internal temperature of the egg provided an approximation of the temperature of the embryo but could not indicate an exact value since at no time was the thermocouple inserted into the embryo.

The initial cooling rates for the eggs were fairly similar at 7.2°C., 5.0°C. and 3.8°C. About two-thirds of the entire temperature drop occurred in the first hour for all temperatures, with the final drop taking about 2 hours more at 7.2°C. 2.5 hours more at 5°C. and 3 hours at 3.8°C. (See Figure 10). From then on the internal temperature of the egg fluctuated within 1 degree of that of the cooler. In Experiments 4 and 5 the egg temperature appeared about a degree centigrade higher than that of the cooler at all times after the low plateau was reached. This variation was slightly greater and more constant than found in any of the other Experiments. This was probably due to the use of the open water bath for recording the temperature of the cooler. As described in Methods, the thermocouples in the open water bath recorded the temperature 0.5 to 1 centigrade degree too low, therefore the actual temperature of the cooler for Experiments 4 and 5 are probably slightly low. The uniformity in rate of cooling of the embryos and the decline in internal temperature to the level of the cooler during treatment at both the early and late stages of incubation would support Romijn and Lokhorst's (24) argument that the chick embryo behaves basically as a poikilotherm until after hatch.

The hatchability of all groups that underwent temperature stress was lower than the controls, usually to a statistically significant degree ($P < .05$). In Experiments 1, 2 and 3 where the eggs were exposed to 7.2°C. $\pm$ 1 for 24 hours commencing at 4 days (96 hours) of incubation, the reduction in hatchability was due to the embryonic mortality which occurred during or shortly after treatment. Hatchability of the treated groups in Experiment 1 (77%) was higher than those found in Experiments 2 (60%) and 3 (35%). The difference may have been due to the different method of exposing the eggs in the cooler, or may be due to differences in strain susceptibility as a commercial broiler strain was used in Experiment 1 versus a commercial White Leghorn hybrid in Experiments 2 and 3. The extremely low hatchability of Experiment 3 may have been caused by the cooler malfunction. Possibly the temperature of the cooler dropped considerably before or during the breakdown of the cooler, but as the temperature recorder was also out of order this can not be established. The cause of the embryonic mortality was probably due to the disruption of physiological processes or damage at the cellular level due to the cold stress. The abnormal development of the limbs of 10 to 15% of the chicks in Experiments 2 and 3 would indicate damage to the cells, specifically in the apical growing region of the limb bud. Up to 50 per cent of the limb bud may be removed between the 4th and 6th days of incubation, the period when limb differentiation and development are at a peak of activity, without altering the morphology of the resulting limb, provided that the distal or apical region are not injured or removed (23). Sturkie (26) has also reported that low temperature stress at the 5th day of incubation has caused similar leg deformities. The absence of limb malformations in Experiment 1 may be

due to a difference in susceptibility of the broiler strain at this stage of incubation. The rate of development of this broiler strain was slightly slower than that of White Leghorn stock as the broiler controls usually peaked hatching about 12 hours later than the White Leghorn controls. It also may have been due to a slower rate of cooling due to different exposure methods. Moreng et al. (15) have shown that a faster rate of cooling was more detrimental to the embryo. However, the low temperature stress may still have affected the limbs in Experiment 1 as the incidence of perosis was higher in the birds treated at 4 days than for the controls. It was also noted (for the limb abnormalities) that usually only one limb was affected, that the left limbs of the chicks were affected most often and that the hind limbs were affected more often than the wing. At four days of incubation the hind limbs are slightly further from the center of the egg than the wing buds and perhaps received a harsher exposure or the leg buds may be more susceptible to cold stress at this stage. Neither position of the egg during treatment, i.e. horizontal versus vertical, nor length of exposure (23 to 25 hours) seemed to affect the rate of abnormalities but those exposed at the latest stage of incubation (98 hours) did have fewer abnormalities.

Buckland (1, 2, 4) reported no reduction in per cent hatchability for eggs cooled at 5°C. $\pm$ 1 for 4 or 8 hours at 17 days of incubation. In this study, 8 hour exposure at 3.8°C. and at 5°C. after 17 days of incubation resulted in no hatchability. Four hours of exposure at either temperature also lowered the hatchability although not always significantly. However, if the hatchabilities from all the four hour exposures at 5°C. are pooled and analyzed (Table 24), the per cent

hatchability of these treatments was significantly (P .05) lower than for the controls. Buckland (3) has had similar results when a different cooler was employed and the temperature also dropped to 3.8 - 4°C. (38.0 - 39°F.). Even advancing the commencement of treatment by six hours to more closely match the times used by Buckland (3) did not greatly increase hatchability. As three different coolers, two by Buckland (1, 2, 3), and one by this author, have been used to stress embryos at 17 days for from 4 to 8 hours at from 3.8 to 5°C., and only one has not proven detrimental to hatchability, it would appear that the rate of cooling and subsequently the total length of time at the ambient temperature in the cooler are more critical values than merely stating the temperature of cooler and length of exposure. Other factors which should be considered when comparing coolers are air circulation and wind chill, position of cooling device in relation to position of eggs to be treated, manner in which eggs are set in the cooler to insure uniform cooling and the humidity of the cooler. This last factor, humidity, was only recorded in Experiments 8 and 9 (R. H. of 12-17%). In normal incubation at 37.5°C. a fairly high relative humidity of 60-70% is desirable during the later stages (9), thus the low humidity during exposure may have caused additional stress and heat loss due to evaporation.

The cause of the low hatchability for embryos exposed to 3.8°C. - 5°C. at 17 days for 4 to 8 hours appears to be a failure of embryos to pip and hatch rather than the occurrence of death during or shortly after treatment as found in the low temperature treatment at 4 days. Examination of the unhatched eggs, in Experiment 6, revealed that many of the treated embryos were still alive at 21.5 days and some survived as long as 23 full days of incubation (Experiment 8). However most of

these embryos had not pipped the shell or made any attempt to hatch. The most probable reason for this was the failure of the hatching muscle to function properly but this was compounded by the malposition of many embryos in the egg which renders hatching more difficult (9). Examination of the treated embryos which failed to hatch revealed that most of them had an edematous and/or hemorrhagic hatching muscle (M. complexus), a condition often found in unhatched embryos incubated normally (6, 17, 19) (Figures 3, 4, 5). The exact cause of this pathological condition is still unknown (17) and whether this was the reason that the hatch muscle failed to function or whether this condition merely arises in conjunction with some other upset to the hatching muscle has not been decided. The cold stress may have injured the capillaries of the hatching muscle area which may be particularly sensitive to a low temperature stress at this period of rapid growth of the hatching muscle in preparation for hatching. Capillary injury could allow the colloid fraction of the blood to pass into the tissue which would result in an increase in fluid in the tissue, i.e. edema (7, 10, 29), due to increase in osmotic pressure of tissue fluid. Injury would also allow the passage of leokocytes through the capillary wall, i.e. hemorrhage (29). Rigdon et al. (18) have reported an absence of aldolase and hexokinase in hatching muscles which were edematous and hemmorrhagic and have proposed that because of this, ATP synthesis in such muscle was lowered and muscle failure occurs due to insufficient ATP. The energy demands of the hatching muscle are fairly high from the seventeenth day of incubation onwards as it increases in size and prepares for hatching. Cooling during this period may have increased the demand for energy and depleted the ATP reserves which, as the edema and hemorrhage condition

developed, were not replaced sufficiently due to a lack of aldolase and hexokinase. This could be the cause of the failure of the chick to pip as the hatching muscle could not function.

The reason for the increased incidence in the number of malpositions from exposure to 3.8 - 5°C. for 4 to 8 hours commencing at 17 days is also unknown. The treatment was made at a time when the embryo was assuming its final position for hatching. The temperature stress appears to interfere with this process so that approximately 50% of the dead embryos are in a difficult position for hatching. The normal position after 17 days of incubation is for the beak to be pointed toward, and later (18 days) to puncture, the air cell of the egg (9, 23). Active lung respiration of the embryo commences as soon as the beak enters the air cell, as the embryonic respiratory system can not meet all the requirements of increased activity of the embryo (23). Malpositions with the head in the small end (away from the air cell) or with the head and beak half way down the egg between the thighs do not allow for the normal switch in respiration to take place and this possibly caused anoxia and may account for some of the embryonic mortality. The cause of these malpositions may be an avoidance reaction occurring during treatment. The percentage of malpositions per number of dead embryos is roughly the same for a four hour treatment as for an 8 hour treatment, around 50%, and seems to be present at the same level at any stage of incubation after treatment (Table 14). This suggests that possibly the malposition occurred during treatment within the first four hours while the egg was cooling. The hatching muscle which is in a fairly active state at this stage may be quite sensitive to cold temperature i.e. pain threshold similar to cramp in pitchers arm which is allowed to cool. It

is possible that the lower half of the egg, protected and insulated by the fibre egg tray from the cold air blast of the cooler fans, was warmer for a period of time than the upper end of the egg and the embryo tried to relieve the stress by seeking the bottom of the egg.

Heart failure could also possibly explain the occurrence of edema and the malpositions of the embryos. When the embryos with the thermocouples were checked for viability, the heart rate was well below normal 200 beats per minute (27) for a developing embryo. Hypothermia undoubtedly reduced the heart rate in the remaining treated embryos. When the embryos were returned to normal incubation conditions, perhaps the heart never regained proper function. Cardiac failure can lead to the development of peripheral edema over a period of several days (7) as reported for the hatching muscle in these experiments. A reduced heart output may have reduced the oxygen supply to the tissue causing anoxia. This may have happened to parts of the nervous system which resulted in malpositions occurring due to loss of neural control of parts of the body.

However, no matter which mechanism causes the embryonic mortality and failure to hatch, cold temperature stress at both early and late stages of incubation caused reduced hatchability, the amount depending on the rate of cooling and total length of exposure. Embryos were able to withstand longer periods of cold stress during the early stages of incubation. These results are in agreement with those reported in the literature review (1, 9, 12, 15, 28).

The growth response of the chicks from eggs treated at 7.2°C. for 24 hours at 4 days of incubation in Experiment 1 did not occur in Experiments 2 or 3. This may be due to a difference in strain of birds as a broiler strain was used in Experiment 1 and a White Leghorn stock

in Experiments 2 and 3. Although Experiment 3 has a weight range between treatments of 50 g. for males and 90 g. for females their differences were not significant (P .05) and the average of all treated males was only 10 g. greater than the control and for females only 25 g. heavier. This wide range of mean weights was probably due to the small number of chicks per treatment which resulted from the high embryonic mortality.

The stress of embryos at 3.8°C. commencing at 17 days of incubation had no effect on post-hatch mean body weights from 0 to 8 weeks at 4, 5 or 8 hours of exposure. However, exposure to 5°C. for 4 hours commencing at 17 days of incubation did affect the mean body weights of male chicks. In Experiment 7, the treated males weighed significantly less (P .05) than the controls at 4 and 8 weeks of age. However in Experiment 8 the treated males weighed significantly more (P .05) than the controls. In Experiment 9, the males from the 4 hour treatment done at 17 days + 3 hours of incubation were 30 g. heavier than the controls and those treated at 16 days + 21 hours of incubation but the difference was not significant. There were never any significant differences between the control and treated females and very little variation. In Buckland's work (1, 2) a significant growth response to the 5°C. temperature stress was also usually greatest for the male chicks. Exposure of embryos at 5°C. commencing at 17 days of incubation although not consistent in the experiments done in this study, does seem to affect the growth response after hatching. This temperature perhaps has some effect on the production of hormones which control growth, particularly the androgens as the males are the sex predominantly affected. Buckland (2) has suggested that growth response was due to elimination of potentially poor chicks before hatch. However, since a similar stress at 3.8°C. produced no

change in growth pattern, as the growth response was predominantly in male chicks, and since an interaction between the light environment and growth response to the temperature stress (4) has been observed, the proposition of a hormonal influence appears more likely.

The inconclusiveness of the studies at 5°C. would indicate that further studies in this field should be carried out. A slower rate of cooling and less total exposure to ambient temperature of cooler may produce results closer to those of Buckland i.e., growth promotion and very little reduction in hatchability. If growth could be stimulated by this means onsistently, with little reduction in hatchability, it could prove to be an economic factor in meat production. Cold temperature stress studies have also been proposed as a means of genetic selection. However this study indicates that for repeatability, there must be adequate control and surveillance of the treatment parameters to insure proper interpretation of results. The physiological and anatomical results of the cold tem perature mortalities may provide useful information. Rigdon et al. (17, 18) have studied the hatching muscle in the edematous and hemorrhagic state as reported here in connection with muscular dystrophy studies in humans. This condition is often the only morphological abnormality in many of the embryos which fail to hatch in normal incubation. Understanding and elimination of these losses, if this is the cause of the hatch failure, could represent substantial savings in hatchery operations. Studies on temperature stress of embryos and the post-hatch growth response could also prove valuable guides to wild fowl conservation studies especially in cases where eggs are taken from the nest to be artificially incubated.

SUMMARY AND CONCLUSIONS

Eggs from two breeds of domestic chickens, Gallus gallus, a commercial broiler breed and a White Leghorn breed, were subjected to a temperature stress of 7.2°C. for 24 hours commencing at 4 days of incubation or to 3.8°C. or 5°C. for periods of from 4 to 8 hours commencing at 17 days of incubation. Records were kept for hatchability, embryonic mortality, morphology, body weight from 0 to 8 weeks, sex and post-hatch mortality. The temperature of the cooler and internal temperature of the egg were recorded during all but two experiments.

The exposure of eggs to 7.2°C. for 24 hours after four days of incubation lowers the hatchability of the eggs. The treatment causes malformation of the limbs when eggs from a White Leghorn stock were treated but no malformation when eggs from a broiler strain were used. This could indicate a genetic difference in susceptibility to this treatment, which could also account for the positive post-hatch growth response in the broiler strain.

Exposure of eggs to 3.8°C. or 5°C. for 8 hours after 17 days of incubation reduced hatchability to zero. Exposure under the same conditions for 4, 5 or 6 hours reduced hatchability proportionally by approximately 25 to 70% below the level of the controls.

All cold temperature stress commencing at 17 days of incubation caused the occurrence of a high incidence of mal-position, usually with the head found in the small end of eggs or down between the thighs. These same treatments caused the occurrence of an edematous and hemmorrhagic

hatching muscle in the majority of the dead embryos.

The temperature stress of 3.8°C. did not affect the post-hatch growth rate of treated embryos. The temperature stress of 5°C. may have an influence on the post-hatch growth response of treated male embryos.

Results indicate that eggs should be separated by at least the width of an egg to allow uniform cooling of all eggs. The rate of cooling of the eggs subjected to 7.2°C., 5.0°C. or 3.8°C. was quite similar at both early and late stages, of incubation.

The factors that should be stated in a temperature stress study are 1) temperature of cooler, 2) rate of cooling of internal temperature of egg, 3) length of time embryo spent at or parallel to temperature of the cooler, 4) relative humidity of cooler. Other factors which would be useful to know are size of cooler and air velocity in cooler.

Table 1

Effect of exposure of embryos to 7.2°C±1 for 24 hours commencing at 4 days (96 hours) of incubation or to 3.8°C±1 for 4 or 8 hours commencing at 17 days (408 hours) of incubation on hatchability, embryonic mortality and morphology

Experiment 1

Treatment	% Hatch[1]	Embryonic Mortality[2]			Malpositions of the Embryo	Number of Occurrences of Edema and Hemorrhage
		0-8 days	9-17 days	17+ days		
Controls 0 exposure	91.4	3	3	3	1	1
24 hours at 7.2°C at 4 days	74.4	19	3	5	2	2
4 hours at 3.8°C at 17 days	77.0	6	0	17	10	11
8 hours at 3.8°C at 17 days	16.1	6	10	71	35	41

[1] the mean hatchability of 8 hour exposure at 17 days is significantly lower than all other treatments (P.05). Hatchability was calculated as per cent of fertile eggs.

[2] embryonic mortality refers to the number of embryonic deaths occurring per treatment from 0-8 days, 9-17 days or after 17 days of incubation.

Table 2

Effect of exposure of embryos to 7.2°C±1 for 24 hours at 4 days of incubation and effect of exposure to 3.8°C±1 for 4 or 8 hours at 17 days of incubation on mean body weights - Experiment 1

Temperature	--	7.2°C	3.8°C	3.8°C
Stage of Incubation	Control	Day 4	Day 17	Day 17
Length of Exposure	--	24 hour	4 hour	8 hour
Hatch Weight[1]	43.37_a	45.2_b	42.57_a	42.1_a
4 Week Weight[2]	607.9	643.9	619.5	631.3
8 Week Weight[3]	1650.75	1726		

[1]Means with the same subscript are not significantly different - Duncan's Test (P.05).

[2]No significant difference (P.05).

[3]Means significantly different by analysis of variance (P.05).

Table 3

Duncan's test[1] showing the significant differences between mean hatch weights of chicks from embryos which received no treatment, of chicks which were exposed commencing at 4 days of incubation to 7.2°C for 24 hours and of chicks which were exposed at 17 days of incubation to 3.8°C for 4 or 8 hours

	4 days 7.2°C 24 hours	Control	17 days 3.8°C 4 hours	17 days 3.8°C 8 hours	P	R	S	RS	Diff.
Mean Hatch Weights	45.2	43.4	42.6	42.1					
					3	3.3	.473	1.56	1.3

[1]Means underscored by same line are not significantly different (P.05).

Table 4

Ambient air temperature in the cooler and the internal temperature of the eggs during treatments for Experiments 2 - 9

Hours of Treatment	7.2°C±1 Exposure				3.8°C±1 Exposure						5°C±1 Exposure						
	Experiment 2		Experiment 3		Experiment 4		Experiment 5		Experiment 6		Experiment 7		Experiment 8		Experiment 9		
	Cooler	Egg	Cooler	Egg	Cooler	Egg	Cooler	Egg	Cooler	Egg	Cooler	Egg	Cooler	Egg	Cooler	Egg 1	Egg 2
0	7.5	37.5	7.0	37.5	4.0	37.5	3.7	37.5	4.0	37.5	5.0	37.5	4.8	37.5	5.0	37.5	37.5
0.5	7.5	21.5	7.5	18.6	4.0	18.6	5.0	21.0	5.0	22.0	5.2	20.5	4.8	22.3	5.0	19.5	23.0
1.0	7.3	14.0	7.5	13.7	3.8	12.7	3.3	12.6	3.8	13.3	5.2	12.6	4.8	13.0	5.0	12.0	13.0
1.5	7.5	11.0	7.5	11.0	3.7	9.3	3.3	8.6	3.8	9.5	5.2	9.0	5.0	9.0	5.0	8.3	8.5
2.0	7.5	8.6	7.7	10.0	3.7	7.6	3.3	6.6	4.0	7.3	5.2	7.5	5.0	7.0	5.0	6.5	6.8
2.5	7.5	7.8	7.5	8.3	3.7	6.3	3.3	5.5	4.0	6.3	5.2	6.0	5.0	6.0	5.0	6.0	5.5
3.0	7.7	7.7	N/A	N/A	3.7	5.7	3.0	5.0	4.0	5.5	5.2	5.5	5.0	5.5	5.0	5.5	5.3
3.5	7.5	7.5			3.7	5.3	3.0	4.7	4.0	5.0	5.2	5.3	5.0	5.3	5.0	5.3	5.0
4.0	7.5	7.5			3.7	5.0	3.0	4.5	4.0	5.0	5.5	5.5	5.0	5.0	5.0	5.0	5.0
4.5	7.5	7.4			3.7	4.7	3.0	4.4	4.2	5.0	5.2	5.2	5.0	5.0	5.0	5.0	5.0
5.0	7.3	7.4			3.5	4.4	3.0	4.4	4.2	4.5	5.2	5.2	5.0	5.0	5.0	5.0	5.0
5.5	7.5	7.3			3.5	4.4	3.0	4.4	4.0	4.5	5.0	5.0	5.0	5.0	5.0	5.0	5.0
6.0	7.3	7.3			3.7	4.6	3.0	4.4	4.0	4.5	5.0	5.0	4.8	4.8	5.3	5.2	5.0
6.5	7.5	7.4			3.7	4.6			4.0	4.5	5.0	5.0	4.8	4.8	5.0	5.0	5.0
7.0	7.3	7.6			3.7	4.6			4.0	4.5	5.0	5.0	4.8	4.8	5.0	5.0	5.0
7.5	7.3	7.5			3.5	4.5			4.0	4.5	5.0	5.0	4.8	4.8	5.0	5.0	5.0
8.0	7.3	7.6			3.5	4.4			4.0	4.5	5.0	5.0	4.8	4.8	5.0	5.0	5.0
12.0	7.5	7.7															
16.0	7.7	7.6															
20.0	7.7	7.8															
24.0	7.7	7.7	10°C														

Table 5

Effect of 24 hour exposure to 7.2°C commencing at 4 days of incubation on hatchability, embryonic mortality and occurrence of deformed embryos and chicks - Experiment 2

Treatment	% Hatch[1]	Embryonic Mortality			Number of Deformed Dead Embryos	Number of Deformed Chicks Hatched
		0-8	9-17	17+		
Controls	85.7	5	1	4	0	0
24 hour - 7.2°C after 4 days incubation	60.5	21	2	4	2 legs 1 wing 3 total	3 wings 2 legs 8 feet 13 total

[1]Hatchabilities significantly different at P.05 level.

Table 6

Effect of 24 hour exposure to 7.2°C at 4 days of incubation on mean body weight at hatch, 4 and 8 weeks and on post-hatch mortality - Experiment 2

Treatment	Mean Body Weight[1] in Grams					Mortality Number of Birds	
		4 weeks		8 weeks			
	Hatch	Males	Females	Males	Females	0-4 week	4-8 week
Controls	37.8	292	272	777	667	4	0
7.2°C for 24 hours after 4 days incubation	38.7	284	251	776	628	2	0

[1]No significant difference in mean body weights. (P .05)

Table 7

Effect of exposure of embryos to 7.2°C. for 23, 24 or 25 hours commencing at 94, 96 or 98 hours of incubation on hatchability - Experiment 3

Hours of Incubation	98	98	96	96	98	94	96	94	96	0
Hours of Exposure	25	24	25	24[1]	23	25	24	24	23	0
% Hatch (Average)	19	21.3	25.6	26.6	32.6	41	39	53	55.6	81.6
Arc si ne (Average)	25.6	27.2	30.3	31	34.7	34.7	38.3	46.7	49.6	64.7

Duncan's Test[2]

P	S	R	RS	Difference
8	7.04	3.36	23.6	21.1
3	7.04	3.1	21.8	18
8	7.04	3.36	23.6	22.4

[1]This 96 - 24 treatment was treated with the long axis of the egg horizontal whereas all other treatments were done vertically.

[2]Means underscored by the same line are not significantly different (P.05) Duncan's test.

Table 8

The effect of exposure of embryos to 7.2°C for 23, 24 or 25 hours commencing at 94, 96 or 98 hours of incubation on embryonic mortality, post-hatch mortality and the incidence of abnormally formed chicks and embryos - Experiment 3

Hours of Incubation before Treatment	Number of Hours Treated	Embryonic Mortality			Post-Hatch Mortality		Number and Types of Abnormalities	
		0-8	9-17	17+	0-4 week	4-8 week	Dead Embryos	Chicks Hatched
94	24	19	1	0	2	2		2 L wings
94	25	20	1	0	3	0		2 legs
96	23	16	1	2	0	1		1 wing 1 leg
96	24	25	1	0	5	0		1 foot
96	24[1]	31	0	0	1	2		2 wings
96	25	29	1	1	0	0	1 wing and foot	2 L wings
98	23	24	1	3	7[2]	0		1 foot
98	24	27	0	5	3	0		1 foot
98	25	27	1	2	1	0	1 leg	
0	0	2	5	0	3	2		

[1]This group was chilled with the long axis of egg in a horizontal position versus the normal vertical position.

[2]5 of these chicks were in the same pen and died when water was unavailable for 3 days after hatch.

Table 9

Effect on hatch weight of exposure of embryos to 7.2°C. for 23, 24 or 25 hours commencing at 94, 96 or 98 hours of incubation; a partial Duncan's table shows significant differences between treatments[1] - Experiment 3

Hours of Incubation	96	98	94	98	98	0	96	96[2]	86	94
Hours of Exposure	23	23	25	23	24	0	25	24	24	24
Hatch Weight	34.3	35	35.5	35.8	36.8	36.8	37.2	37.2	37.5	37.7

P	R	S	RS	Difference	Significance
9	3.29	.884	2.9	2.7	No
5	3.12	1.06	3.2	2.5	No

[1]Means underlined by same line are not significantly different (P.05) Duncan's test.

[2]Eggs in this treatment were treated with the long axis horizontal.

Table 10

Effect of exposure of embryos to 7.2°C for 23, 24 or 25 hours after 94, 96 or 98 hours of incubation on mean body weights[1] - Experiment 3

Treatment		Mean Body Weights in Grams			
Number of Hours of Incubation Prior to Treatment	Number of Hours of Treatment	4 weeks		8 weeks	
		Male	Female	Male	Female
94	24	291	297	790	692
94	25	349	296	809	693
96	23	324	269	827	638
96	24	331	307	804	731
96	24[2]	333	316	799	713
96	25	345	307	851	671
98	23	330	310	797	697
98	24	299	262	790	659
98	25	333	301	800	712
0	0	339	284	798	664

[1]No significant differences between treatments.

[2]Horizontal treatment.

Table 11

Effect of exposing embryos to 3.8°C for 4 and 8 hours commencing at 17 days of incubation, on hatchability, embryonic mortality and morphology
Experiment 4

Hours of Treatment	% Hatch[1]	Embryonic Mortality			Malposition of the Head	Presence and Degree of Edema[2]			Presence and Degree of Hemorrhage[2]		
		0-8	8-17	17+		1	2	3	1	2	3
Controls	92	1	0	3	1	0	1	0	1	1	0
4 Hours	71.5	2	0	13	6	1	5	0	4	2	1
8 Hours	0	3	0	51	23	5	28	9	13	10	2

[1] Significant difference (P.05) between controls and 4 hour treatment. The 8 hour treatment was significantly lower than both the controls and 4 hour exposure.

[2] The values 1, 2, 3 represent a visual scale.

For Edema
1 = 25% increase in fluid and muscle size
2 = 25 to 75% increase in fluid and muscle size
3 = 75% or greater increase in fluid and muscle size

For Hemorrhage
1 = 5 to 20% of muscle area covered
2 = 20 to 40% of muscle area covered
3 = 40% or greater of muscle area covered

Table 11B

Duncan's test for % hatchability for Experiment 4

Length of Exposure	8 Hour	4 Hour	0 Hours	P	R	RSy	Difference
% Hatch	0	71.5	92				
Arcsin Transformation[1]	7.8	57.8	74.6				
				3	3.34	9.41	66.8*
				2	3.2	9.02	50.0*
				2	3.2	9.02	16.8*

*Means significantly different (P .05)

[1]0 per centages were transformed to arcsin values by method in Snedicor (25) p. 328

Table 12

Effect of exposing embryos to 3.8°C for 4 hours after 17 days of incubation, on mean body weights at hatch, 4 and 8 weeks[1] - Experiment 4

Treatment	Hatch Weight gms.		4 week Weight gms.		8 week Weight gms.	
	male	female	male	female	male	female
Control	37.2	35.3	318	278	841	702
4 Hour	35.3	36.6	319	292	872	735

[1]No significant differences between treatments (P.05) at time of hatch, 4 weeks or 8 weeks.

Table 13A

Effect of exposing embryos to 3.8°C for 5 and 6 hours commencing at 17 days of incubation, on hatchability, embryonic mortality and morphology
Experiment 5

Hours of Treatment	% Hatch[1]	Embryonic Mortality			Malposition of the Head	Presence and Degree of Edema			Hemorrhage		
		0-8	9-17	17+		1	2	3	1	2	3
Controls	75.5	6	0	7	3	1	3	0	2	3	0
5 Hours	13	1	2	56	40	11	24	11	13	15	14
6 Hours	5.6	2	5	60	28	6	13	20	19	5	14

[1]Hatchability for 5 and 6 hour treatments are significantly (P.05) lower than controls as shown by Duncan's Test below.

Table 13B

Duncan's Test showing significant difference between treatments[2]

Treatment	Controls	5 Hour 3.8°C Day 17	6 Hour 3.8°C Day 17
% Hatch	75	13	5.6

P	S	R	RS	Difference
2	6.06	3.2	19.4	7.2

[2]Means underscored by same line are not significantly different (P.05).

Table 14

Effect of exposure of embryos at 17 days of incubation to 3.8°C for 0, 4, 5 or 8 hours, on position in the egg and appearance of hatching muscle - Experiment 6

Age of Incubation	Hours of Exposure	Number Embryos Examined	Malposition	Presence and Degree of Edema			Hemorrhage		
				1	2	3	1	2	3
18 Days	0	4	0	0	0	0	0	0	0
	4	4	0	0	0	0	0	0	0
	5	4	2	0	0	0	0	0	0
	8	4	1	0	0	0	0	0	0
19.5 Days	0	4	0	0	0	0	1	0	0
	4	4	1	0	0	0	0	0	0
	5	4	0	4	0	0	2	0	0
	8	4	1	1	0	0	2	0	0
20.5 Days	0	3[1]	-	0	0	0	0	0	0
	4	3[2]	1	0	0	1	0	0	1
	5	3	1	1	1	1	0	2	1
	8	3	1	1	1	1	2	1	0
21.5 Days	0	3[1]	-	0	0	0	0	0	0
	4	3[1]	-	1	0	0	1	0	0
	5	3[3]	1	1	1	1	0	0	2
	8	3	2	0	0	2	1	1	0

[1]All muscles examined from chicks which had hatched and appeared normal.

[2]One muscle examined from one of 4 hour treated chicks which had hatched by 20.5 days.

[3]One muscle sample from a 5 hour treated chick which had hatched by 21.5 days.

Table 15A

Effect of exposure of embryos to 3.8°C for 0, 4, 5 or 8 hours, after 17 days of incubation, on the wet weight of the hatching muscle, expressed as a percent of wet body weight, during 18 to 21.5 days of incubation - Experiment 6

Treatment	Day of Incubation Examined			
	18 Day	19.5 Day	20.5 Day[1]	21.5 Day[1]
0 Hours	.95	1.22	1.04	.86
4 Hours	.88	.99	1.60	1.03
5 Hours	.80	1.39	1.95	2.12
8 Hours	.80	1.20	2.73	3.27

[1]Significant difference in muscle weight between treatments as shown by Duncan's test, Table 15B.

Table 15B

Duncan's test on variations in weights of hatching muscle examined at 20.5 days incubation, expressed as percent of body weight[1]

(a)	0 Hour	4 Hour	5 Hour	8 Hour					
%	1.04	1.6	1.95	2.73					
Arcsine	5.8	7.1	7.3	9.5	P	R	S	RS	Diff.
					3	3.39	.588	1.99	1.5

Duncan's test on variations in weights of hatching muscle examined after 21.5 days incubation, expressed as percent of body weight[1]

(b)	0 Hour	4 Hour	5 Hour	8 Hour					
%	.86	1.03	2.12	3.27					
Arcsine	5.3	5.8	8.0	10.3	P	S	R	RS	Diff.
					3	.883	3.39	2.99	2.7
					2	.883	3.26	2.87	2.3

[1]Means underscored by same line are not significantly different (P.05).

Table 16A

Effect of exposure of embryos to 3.8°C for 0, 4, 5 and 8 hours commencing at 17 days of incubation, on hatchability, embryonic mortality and morphology - Experiment 6

Hours of Treatment	% Hatch[1]	Embryonic Mortality			Malposition of the Head	Presence and Degree of Edema			Hemorrhage		
		0-8	9-17	17+		1	2	3	1	2	3
Controls	86.5	2	2	2	0	0	0	0	0	0	0
4 Hours	68.9	0	0	14	12	4	5	4	1	3	8
5 Hours	16.9	3	0	33	20	3	10	11	1	6	17
8 Hours	0	0	2	40	25	11	14	11	10	10	3

[1] A significant difference (P .05) was found between treatments. Duncan's test (Table 16B) showed the 8 hour treatment was significantly lower than all other groups while the 5 hour treatment was significantly lower than the 0 and 4 hour treatments and the 4 hour treatment was not significantly different than the controls.

Table 16B

Duncan's test for % hatchability for Experiment 6[1]

	8 Hour	5 Hour	4 Hour	0 Hour				
% Hatch	0	16.9	68.9	86.5				
Arcsin Transformation[2]	8.9	24.1	57.7	68.8	P	R	RSy	Difference
			———		2	3.08	13.5	11.1

[1]Means underscored by same line are not significantly different (P .05)

[2]0 per centages transformed to arcsin values by method quoted in Snedicor (p) p. 328

Table 17

Effect of exposure of embryos to 3.8°C for 0, 4 or 5 hours at 17 days of incubation on mean body weights[1] and post-hatch mortality - Experiment 6

Treatment	Hatch Weight gms.		4 week Weight gms.		8 week Weight gms.		Post-Hatch Mortality	
	male	female	male	female	male	female	0-4 weeks	4-8 weeks
0 Hours	33.7	32.3	296	279	777	668	0	0
4 Hours	33.6	32.7	301	276	797	667	0	0
5 Hours	33	31	275	245	742	622	0	0

[1]No significant differences (P.05) among treatments.

Table 18A

Effect of exposure of embryos to 5°C±1 for 0, 4 or 8 hours, commencing at 17 days of incubation, on hatchability, embryonic mortality and morphology - Experiment 7

Hours of Treatment	% Hatch[1]	Embryonic Mortality			Malposition of the Head	Presence and Degree of Edema			Hemorrhage		
		0-8	8-17	17+		1	2	3	1	2	3
Controls 0	88.9	3	1	2	0	0	0	0	0	0	0
4 Hours	66.6	6	0	12	9	3	5	1	1	1	4
8 Hours	0	2	6	45	32	7	17	19	15	12	5

[1] A significant difference (P.05) between 0 and 4 hour exposure, 0 and 8 hour exposure and 4 and 8 hour exposure as shown by Duncan's Test Table 18B.

Table 18B

Duncans test for % hatchability for Experiment 7

Length of Exposure	8 Hours	4 Hours	0 Hours				
% Hatch	0	66.6	88.9				
Arcsin Transformation	7.6	55.4	71.1	P	$	RSy	Difference
				3	3.34	15.8	63.5*
				2	3.20	15.1	47.8*
				2	3.20	15.1	15.7*

*All treatments significantly different (P .05)

Table 19

Effect of exposure of embryos to 5°C±1 for 0, 4 or 8 hours, commencing at 17 days of incubation, on mean body weights and post-hatch mortality - Experiment 7

Treatment	Hatch Weight gms.		4 week Weight gms.		8 week Weight gms.		Post-Hatch Mortality	
	male	female	male[1]	female	male[1]	female	0-4 weeks	4-8 weeks
Control 0	38.9	38.6	293.5	252.4	770.9	635	1	1
4 Hours	37.4	37.8	263.1	255.4	708.6	624	5	0

[1]Control males significantly heavier than treated males (P.05).

Table 20

Effect of exposure of embryos to 5°C±1 for 0, 4 or 8 hours, commencing at 17 days of incubation, on hatchability, embryonic mortality and morphology - Experiment 8

Hours of Treatment	% Hatch[1]	Embryonic Mortality			Malposition of the Head	Presence and Degree of Edema			Presence and Degree of Hemorrhage		
		0-8	8-17	17+		1	2	3	1	2	3
Controls 0	83	5	1	5	0	0	0	0	0	0	0
4 Hours	63	3	1	25	17	5	7	5	5	7	6
8 Hours	0	8	9	58	38	6	16	25	21	6	4

[1]No significant difference (P.05) between 0 and 4 hour exposure.

Table 20B

Duncan's test for % hatchability for Experiment 8

Length of Exposure	8 Hours	4 Hours	0 Hours				
% Hatch	0	63	83				
Arcsin Transformation	7.0	53.2	67.7	P	R	RSy	Difference
				2	3.2	16.3	14.5*

*Means underscored by same line are not significantly different (P .05)

Table 21

Effect of exposure of embryos to 5°C for 0 or 4 hours, commencing at 17 days of incubation, on mean body weights and post-hatch mortality - Experiment 8

Treatment	Hatch Weight gms.		4 week Weight gms.		8 week Weight gms.		Post-Hatch Mortality	
	male	female	male[1]	female	male[1]	female	0-4 weeks	4-8 weeks
Control 0	35.9	34.8	270.7	262.6	707	645	1	0
4 Hour	35.9	36.2	297.7	262.5	780	655	2	0

[1]Treated males significantly heavier than control males (P .05)

Table 22

Effect of exposure of embryos to 5°C for 0, 4 or 8 hours, commencing at either 16 days + 21 hours or 17 days + 3 hours of incubation, on hatchability, embryonic mortality and morphology - Experiment 9

Hours of Treatment	% Hatch[1]	Embryonic Mortality			Malposition of the Head	Presence and Degree of Edema			Hemorrhage		
		0-8	8-17	17+		1	2	3	1	2	3
Controls 0	90.0	2	0	3	0	1	0	0	0	0	0
16 days + 21 hrs. 4 hour exposure	55.5	5	0	19	7	0	5	5	2	3	2
17 days + 3 hours 4 hour exposure	42.9	1	0	33	20	13	4	7	6	3	10
16 days + 21 hrs. 8 hour exposure	0	2	3	49	28	6	9	17	14	2	1
17 days + 3 hours 8 hour exposure	0	1	3	54	36	11	15	19	7	0	1

[1] A significant difference (P.05) between 0 and 4 hour exposure at both stages of incubation. No significant difference between stages of incubation. The 8 hours exposure was significantly lower hatch than 0 or 4 hour exposure.

Table 22B

Duncan's test % hatchability for Experiment 9

Length of Exposure	8 Hour	8 Hour	4 Hour	4 Hour	0 Hour
Stage of Incubation	17 day + 3hr	16 day + 21hr	17 day + 3hr	16 day + 21hr	0 day
% Hatch	0	0	42.9	55.5	90
Arcsin Transformation	6.47	6.75	40.4	48.4	72.7

P	R	RSy	Difference
2	3.15	19.3	0.18*
2	3.15	19.3	8.0

*Means underscored by same line are not significantly different (P .05)

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Table 23

Effect of exposure of embryos to 5°C for 0 or 4 hours, commencing at 16 days + 21 hours or 17 days + 3 hours of incubation, on mean body weights[1] at time of hatch and at 7 and 8 weeks - Experiment 9

Treatment	Hatch Weight gms.		4 week Weight gms.		8 week Weight gms.		Post-Hatch Mortality	
	male	female	male	female	male	female	0-4 weeks	4-8 weeks
Controls 0 hours	33.5	33.2	301	258	744	631	3	0
16 days + 21 hrs. 4 hour exposure	34.1	33.4	297	268	742	668	6	1
17 days + 3 hours 4 hour exposure	35.0	33.9	321	259	777	624	0	1

[1]No significant difference in mean body weights (P .05)

Table 24A

Effect of exposure of embryos to 5°C for 4 hours, commencing at 17 days + 3 hours of incubation, on hatchability in Experiments 7, 8 and 9

Treatment*	% Hatchability Experiment 7	Experiment 8	Experiment 9
Controls	88.9	83.3	90
4 hours at 5°C - 17 days + 3 hours incubation	66	63.2	42.9

Table 24B

Analysis of variance for pooled data for hatchability of 4 hour exposure group from Experiments 7, 8 and 9
The data from the 4 hour exposure at 16 days + 21 hours was excluded as the time of exposure was different

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square
Total	21	6382.39	
Experiment	2	146.79	73.39
Treatment	1	2644.4	2644.4 *
Trt. x Exp.	2	422.0	211.4
Error	16	3258.11	203.6

*Significant difference between treatments (P.05).

Replicate	E	F	G	H
% Hatch	0	26.9	36	0
No. dead Embryo 17+ days	21	16	12	22

Figure 1. This diagram represents the respective position of replicates within the incubator tray as set in the cooler from the treatment for 8 hours at 3.8°C. at 17 days in Experiment 1.

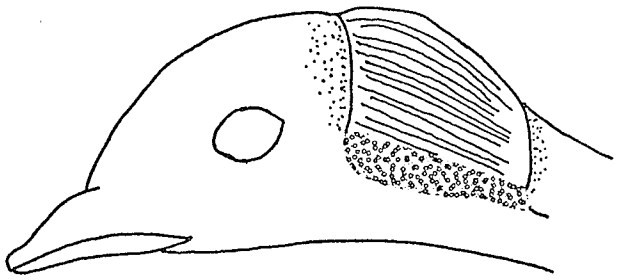

Figure 2. Diagrammatic lateral view of M. complexus (hatching muscle) of the chick at hatching. (after Fisher 1958)

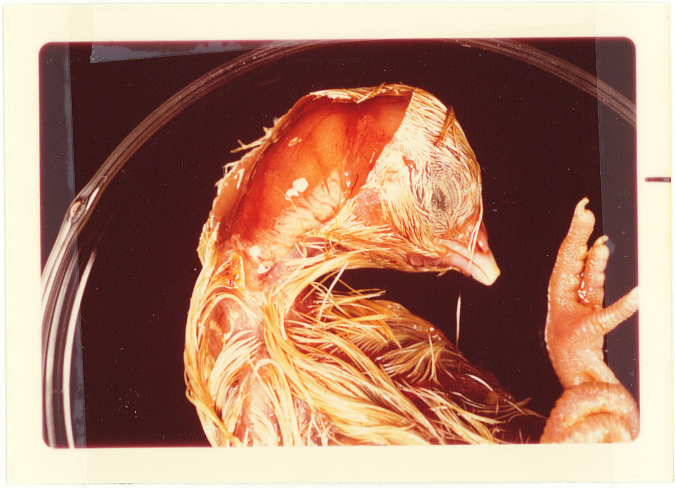

Figure 3. (A) External appearance of embryo with edematous and hemorrhagic hatching muscle

(B) Cut-away view of embryo with edematous and hemorrhagic hatching muscle

Figure 4. Embryo heads illustrating degrees of edema in hatching muscle from embryos cold temperature stressed at 3.8 to 5.0°C. at 17 days of incubation.
Top Left: Normal amount of edema in control embryos
Bottom Left: Degree 1 = 25% increase in edema
Top Right: Degree 2 = 25 to 75% increase in edema
Bottom Right: Degree 3 =75% or greater increase in edema

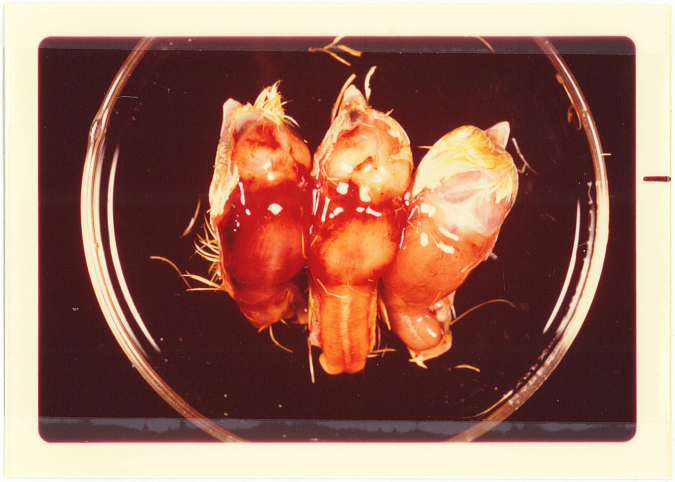

Figure 5. Embryos illustrating degrees of hemorrhage in hatching muscle.
Right: Degree 1 = 5 to 20% of the muscle area covered
Middle: Degree 2 = 20 to 40% of the muscle area covered
Left: Degree 3 = 40% + of the muscle area covered

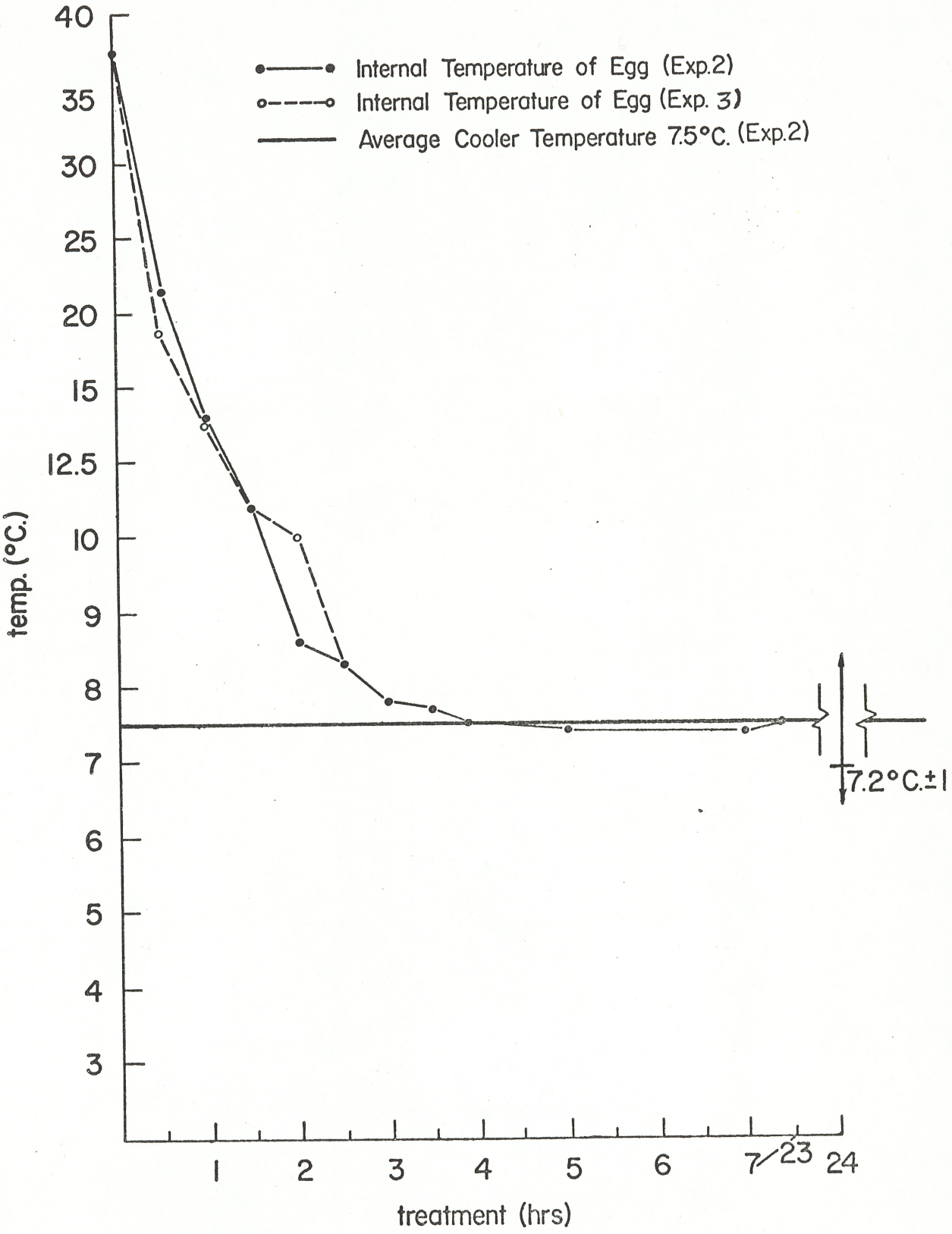

Figure 6. Temperature recordings of the cooler and of the internal egg during treatments set for 7.2°C. ± 1 in Experiments 2 and 3. No record for Experiment 3 after 2.5 hours-recorder failure.

1

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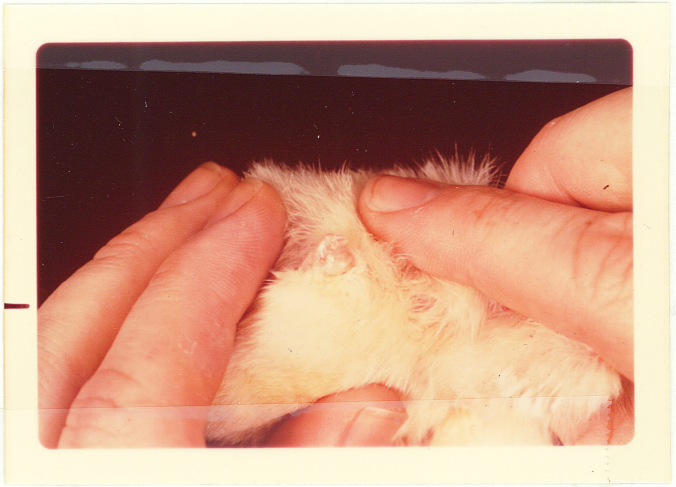

Figure 7. Series of limb deformities caused by exposure of embryos to 7.2°C. Experiments 2 and 3

1. Deformed wing and leg
2. Deformed legs
3. Deformed left leg
4. Deformed leg - only a stub and one toe nail left

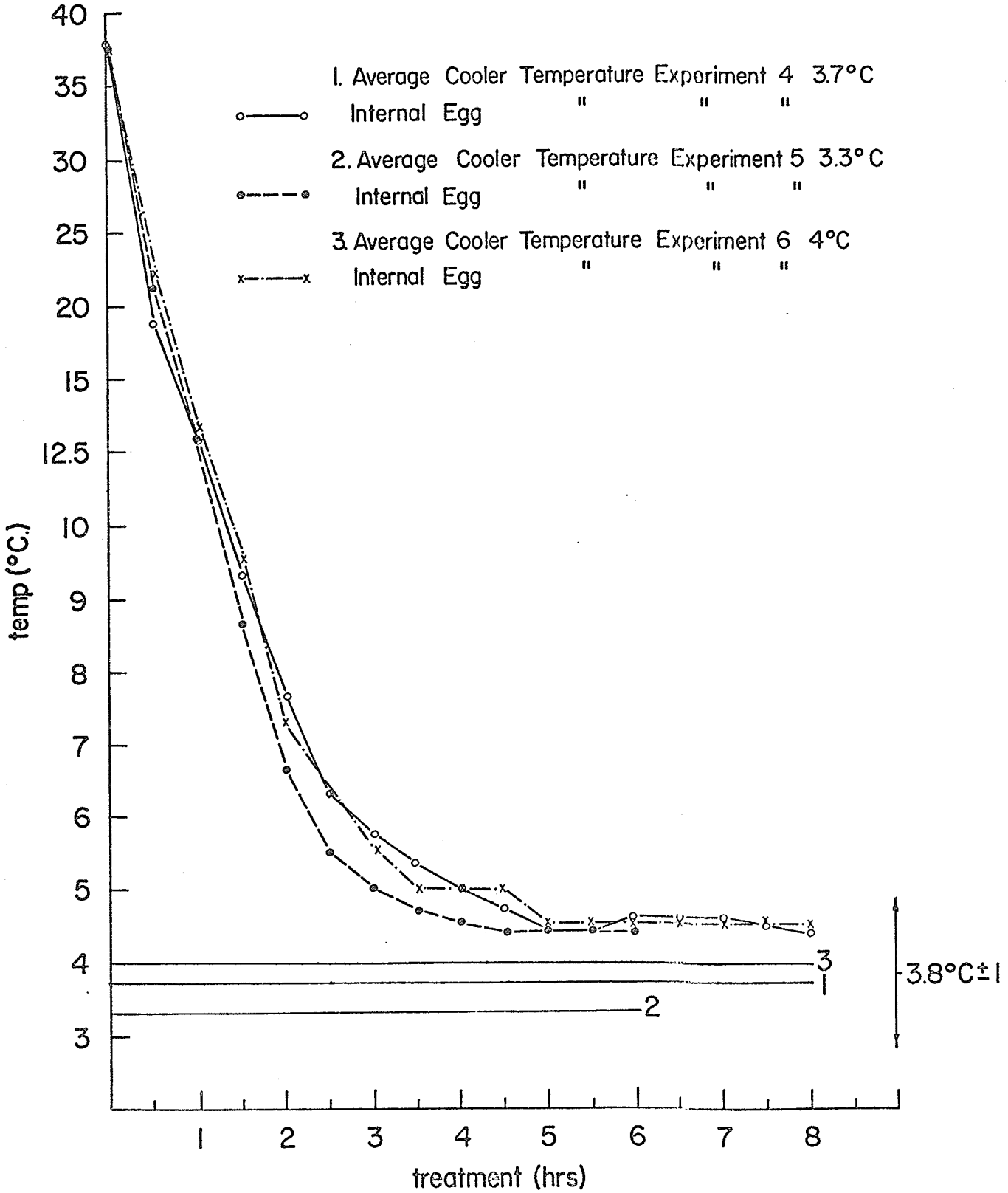

Figure 8. Temperature recordings of the cooler and the internal egg during treatments set for 3.8°C. ± 1 in Experiments 4, 5 and 6.

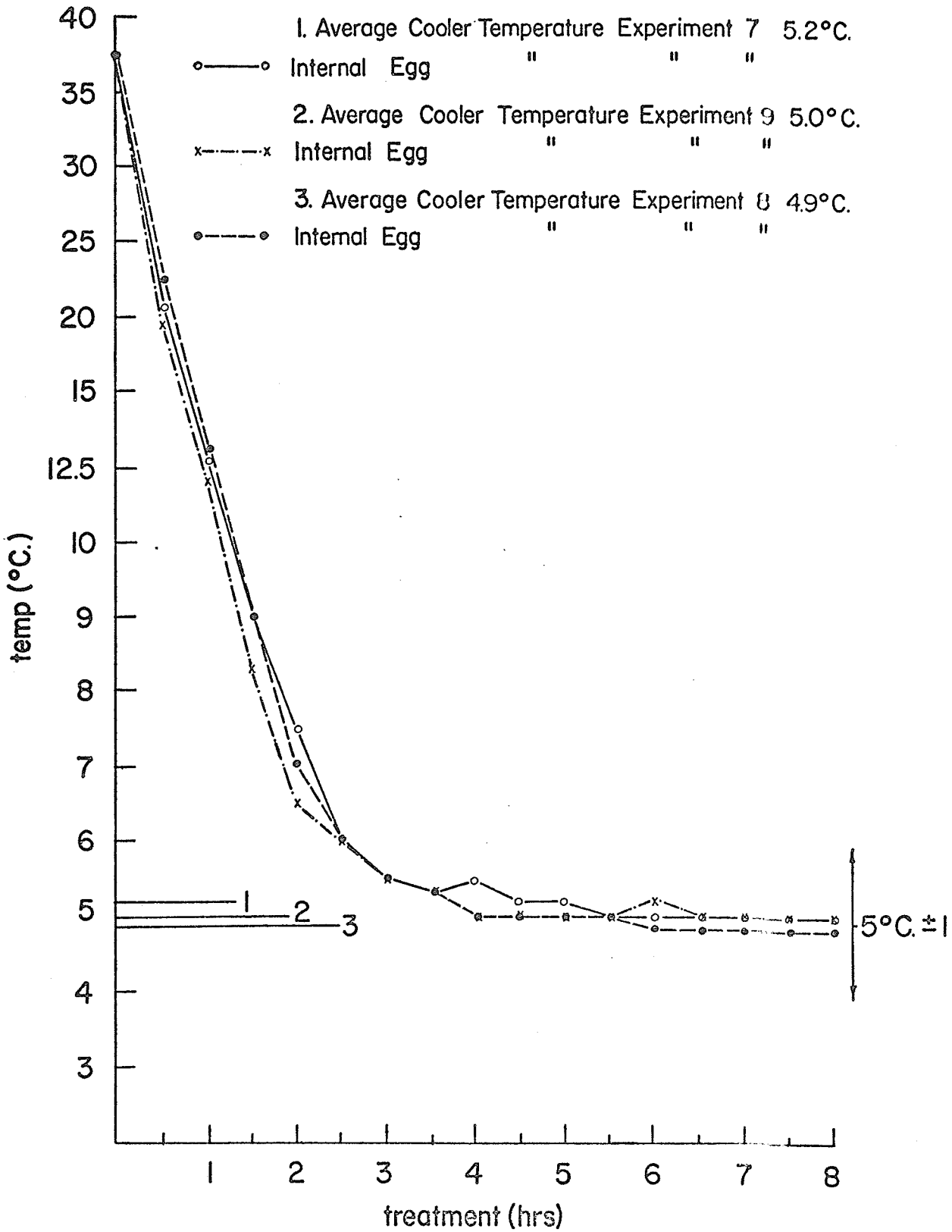

Figure 9. Temperature recordings of the cooler and the internal egg during treatments set for 5.0°C. ± 1 in Experiments 7, 8 and 9.

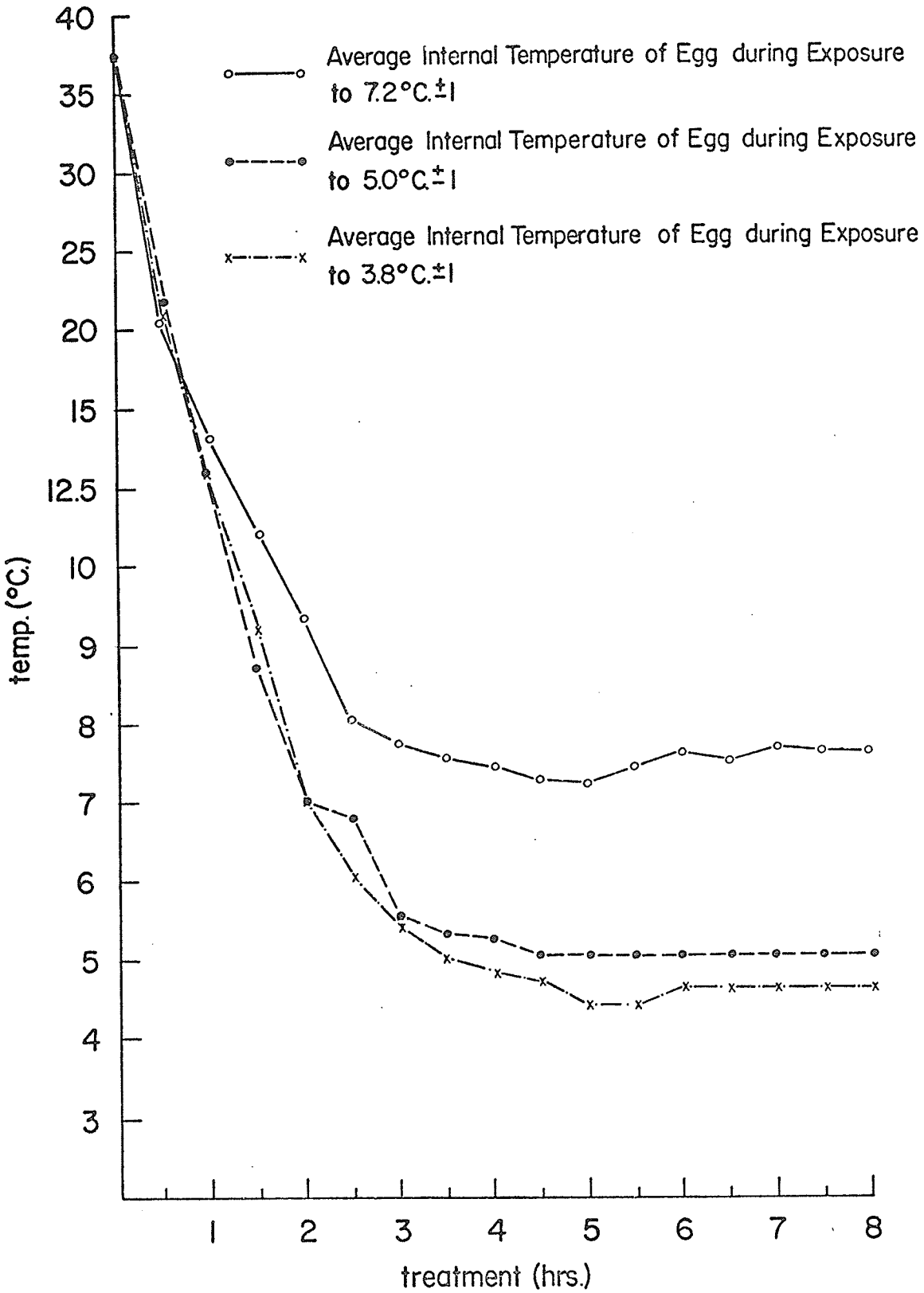

Figure 10. Comparative rates of cooling for eggs subjected to 3.8, 5.0 or 7.2°C.

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