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Factors Affecting the Toxicity of Methylmercury Injected into Eggs

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Abstract. We developed a standardized protocol for comparing the sensitivities of the embryos of different bird species to methylmercury when methylmercury was injected into their eggs. During the course of developing this protocol, we investigated the effects of various factors on the toxicity of the injected methylmercury. Most of our experiments were done with chicken (*Gallus domesticus*), mallard (*Anas platyrhynchos*), and ring-necked pheasant (*Phasianus colchicus*) eggs, all of which were purchased in large numbers from game farms. A smaller amount of work was done with double-crested cormorant (*Phalacrocorax auritus*) eggs collected from the wild. Several solvents were tested, and corn oil at a rate of 1 μl/g egg contents was selected for the final standardized protocol because it had minimal toxicity to embryos and because methylmercury dissolved in corn oil yielded a dose–response curve in a range of egg concentrations that was similar to the range that causes reproductive impairment when the mother deposits methylmercury into her own eggs. The embryonic stage at which eggs were injected with corn oil altered mercury toxicity; at early stages, the corn oil itself was toxic. Therefore, in the final protocol we standardized the time of injection to occur when each species reached the morphologic equivalent of a 3-day-old chicken embryo. Although solvents can be injected directly into the albumen of an egg, high embryo mortality can occur in the solvent controls because of the formation of air bubbles in the albumen. Our final protocol used corn oil injections into the air cell, which are easier and safer than albumen injections. Most of the methylmercury, when dissolved in corn oil, injected into the air cell passes through the inner shell membrane and into the egg albumen. Most commercial incubators incubate eggs in trays with the air cell end of the egg pointing upward, but we discovered that mercury-induced mortality was too great when eggs were held in this orientation. In addition, some species of bird eggs require incubation on their sides with the eggs being rolled 180° for them to develop normally. Therefore, we adopted a procedure of incubating the eggs of all species on their sides and rolling them 180° every hour. Little has been published about the conditions of temperature, humidity, and the movements to which eggs of wild birds need to be subjected for them to hatch optimally under artificial incubation. Not unexpectedly, hatching success in an artificial incubator is generally less than what natural incubation by the parents can achieve. However, the survival of control embryos of most wild bird species was good (generally ≥ 80%) up to within 1 or 2 days of hatching when we incubated the eggs at 37.5°C (or 37.6°C for gallinaceous species) at a relative humidity that resulted in an approximate 15% to 16% loss in egg weight by the end of incubation and by incubating the eggs on their sides and rolling them 180°/h. To improve statistical comparisons, we used survival through 90% of incubation as our measurement to compare survival of controls with survival of eggs injected with graded concentrations of mercury.

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Methylmercury is considered to be the most toxic form of mercury in the environment (Thompson 1996; Wiener *et al.* 2003) and is also the predominant chemical form of mercury reported in the eggs of wild birds (Rumbold *et al.* 2001; Scheuhammer *et al.* 2001). The embryo seems to be the life stage at which birds are most sensitive to methylmercury (Heinz 1979; Scheuhammer 1987; Thompson 1996; Wiener *et al.* 2003). Guidelines to protect avian embryos from mercury poisoning are based largely on a few captive-breeding studies done with chickens (*Gallus domesticus)* (Tejning 1967), mallards (*Anas platyrhynchos*) (Heinz 1979), and ring-necked pheasants (*Phasianus colchicus*) (Fimreite 1971). Although there is no reason to believe that the embryos of all birds are equally sensitive to the harmful effects of methylmercury, the concentrations of mercury shown to be harmful to mallard and pheasant eggs have been used as default values to protect the embryos of wild birds for which no toxicity data are available (Eisler 2000; Henny *et al.* 2002; Meyer *et al.* 1998; Scheuhammer *et al.* 2001; Thompson 1996; Wiemeyer *et al.* 1984; Wolfe *et al.* 1998). Given the great expense and time required to establish captive-breeding colonies of wild birds and feed the adults methylmercury, it is unlikely that many controlled studies will be done to establish toxic thresholds for their eggs. As an alternative to captive-breeding studies, we developed a protocol by which the eggs of wild birds could be brought into the laboratory and injected with graded concentrations of methylmercury chloride. The lot of methylmercury chloride we used was approximately 90% pure, with the

remaining 10% being ethylmercury chloride. Both methylmercury and ethylmercury act primarily as neurotoxins, and the neurologic signs of methylmercury and ethylmercury poisoning have been reported to be very similar (Magos *et al.* 1985). When ethylmercury was fed to breeding ring-necked pheasants at a dietary concentration of 4.2 μg/g mercury, embryo survival was decreased, and egg residues of mercury ranged from 0.9 to 3.1 μg/g wet weight (Spann *et al.* 1972). These egg residues were comparable with those in the eggs of pheasants fed methylmercury, in which embryo survival also was decreased (Borg *et al.* 1969).

During the course of developing a standardized egg injection protocol, we tested the effects of several variables on the embryotoxicity of the injected methylmercury. Our purpose was to learn how to set the test variables such that good dose-response data were generated and the degree of toxicity of the injected mercury was as close as possible to the toxicity of mercury maternally deposited in eggs.

Methods and Results

We studied the following factors: (1) selection of an appropriate toxic end point, (2) orientation and turning of eggs during incubation, (3) choice of solvent, (4) variations in volume of solvent injected, (5) effect of the site where the injection was made, and (6) age of the embryo when the egg was injected. Table 1 lists the species and test conditions used in each of our 36 experiments. Some experiments contained more than one part. We include the results of each experiment under the factor that our experiment was designed to best address, although more than one factor was incorporated into every experiment.

Selection of an End Point for Measuring Mercury Toxicity

With the eggs of wild birds—where optimum conditions of temperature, humidity, and egg turning are unknown—hatching success of artificially incubated eggs can be worse than that of the game farm species. When the artificially incubated eggs of wild birds are candled, the embryos generally appear to be normally developed and vigorous up to within 1 or 2 days of when they are scheduled to hatch, but many will not hatch. Fortunately, because control eggs of most wild birds survive almost to the point of hatching, we were able to use, as a somewhat arbitrary end point, the percentage of embryos that survived through 90% of the incubation period for that species.

Orientation and Turning of Eggs During Incubation

Our incubators, and many other commercial incubators, are built so the eggs sit vertically in plastic trays with the air cell end (cap) of the egg pointing up and the pointed end of the egg (apex) pointing down. The trays of eggs are then typically rotated approximately 30° to 45° by a mechanism that tilts an entire tray first one way and then, an hour later, the other way. We began our injection studies using this vertical positioning of eggs. However, we abandoned the vertical orientation for two reasons. First, we discovered that most species of wild bird eggs survive better when the eggs are allowed to rest in their normal, flat position and are rolled 180° every hour (Harvey 1993). These 180° turns are made clockwise one time and then counterclockwise the next; otherwise the chalazae will become too tightly wound and will harm the egg (Landauer 1967). Second, as will be discussed below in four experiments with mallard eggs, we discovered that when eggs were incubated vertically, the toxicity of methylmercury injected into the air cell was much greater than when the mother deposited the same amount of methylmercury into her eggs.

Experiment 1. One set of 55 mallard eggs was injected with 50 μl pure corn oil (referred to as the "solvent control" in the experimental design detailed in Table 1), another set of 55 eggs was not injected with any corn oil ("control without solvent" in Table 1), and 7 sets of 55 eggs were injected with 50 μl corn oil containing amounts of methylmercury that would result in eggs containing 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 μg/g mercury, respectively, on a wet-weight basis. The injections were made into the air cell of the egg after the eggs had been incubated for 4 days. Fifty μl corn oil was the standard injection volume we used in our early studies with mallards before we adopted the practice of injecting 1 μl solvent/g of egg contents. Because our mallard eggs weighed approximately 50 to 60 g, an injection rate of 50 μl corn oil/egg was close to 1 μl/g egg contents. All eggs were incubated in plastic trays that held the eggs with the air cell end pointing up (vertical). The results from experiment 1 were compared with those from experiment 2.

Experiment 2. The same concentrations of mercury that had been used in experiment 1 were injected. The eggs were injected with 1 μl corn oil/g egg contents. Every hour, the eggs were rolled 180°. In Table 1 we refer to this means of turning the eggs as "rolled 180° with egg on its side."

Regardless of whether the eggs were incubated vertically or on their sides, there were no statistically significant differences in survival between the controls without solvent and the controls injected with pure corn oil (Fisher's exact probability test; $\alpha = 0.05$). However, the survival of the eggs injected with various doses of methylmercury and incubated on their sides was better than the survival of similarly dosed eggs that were incubated in a vertical position (Fig. 1). Regardless of the orientation of an egg, the yolk rotates so the embryo faces up, and when eggs are incubated in the vertical position this rotation of the yolk results in the embryo being directly under the air cell, even when the tray of eggs rotates back and forth. We attribute the greater toxicity of methylmercury to embryos when eggs are situated vertically to the fact that because the methylmercury in a solvent passes through the inner shell membrane and into the albumen of the egg, it is still very concentrated when it makes contact with the embryo, which is sitting right below the membrane. When an egg is incubated on its side the embryo again rotates to an upward-facing position, but this time the embryo floats up to the middle (equator) of the egg and is farther away from the inner shell membrane. Consequently, by the time the methylmercury in the solvent reaches the embryo in an egg resting on its side, it is more diluted.

Experiment 3. Mallard eggs were either controls (injected and uninjected) or were injected with enough methylmercury to achieve a dose of 1.6 μg/g mercury in the egg. The eggs were incubated in a vertical position. The results were compared with those from experiment 4.

Experiment 4. Two sets of mallard eggs were injected in an identical fashion to those in experiment 3, but these eggs were incubated on their sides and were rotated only approximately 60° ("rotated 60° with egg on its side" in Table 1). Survival of the uninjected controls was 88%, which was not significantly different from the survival of the corn oil controls incubated on their sides (100%) or the corn oil controls incubated vertically (86.7%). With the eggs injected

Table 1. List of species and conditions used in egg injection experiments

Experiment No.[a]	Species	Age of embryo (days and hours)	Solvent	Volume of solvent (µl/g of egg contents)	Site of injection	Egg position during incubation	Mercury concentrations injected into eggs (µg/g of egg contents on a wet-weight basis)	Sample size for each group listed
Experiments designed primarily to compare the survival of eggs incubated vertically versus eggs incubated on their sides								
1	Mallard	4d[b]	Corn oil	~1[c]	Air cell	Vertical	Control without solvent, solvent control, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2	All at n = 55[d]
2	Mallard	4d	Corn oil	1	Air cell	Rolled 180° with egg on its side	Control without solvent, solvent control 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2	60 29 to 30[e]
3	Mallard	4d	Corn oil	1	Air cell	Vertical	Control without solvent Solvent control, 1.6	25 29 to 30
4	Mallard	4d	Corn oil	~1	Air cell	Rotated 60° with egg on its side	Solvent control, 1.6	30
Experiments designed primarily to compare the survival of eggs injected with various solvents								
5	Ring-necked pheasant	3d, 7h[f]	Corn oil	1	Air cell	Rolled 180° with egg on its side	Control without solvent Solvent control 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2	50 57 23 to 29
6	Chicken	0d[g]	Propylene glycol	~1	Air cell	Rolled 180° with egg on its side	Control without solvent, solvent control, 4, 16	All at n = 10
7 (part 1)	Mallard	4d	Corn oil	0.5	Air cell	Rotated 60° with egg on its side	Control without solvent Solvent control, 2	30 29 to 30
7 (part 2)	Mallard	4d	Soybean oil	0.5	Air cell	Rotated 60° with egg on its side	Solvent control, 2	29 to 30
8 (part 1)	Mallard	4d	Crisco	0.5	Air cell	Vertical	Control without solvent Solvent control, 4	28 28
8 (part 2)	Mallard	4d	Crisco	1	Air cell	Vertical	Solvent control, 4	28
9	Chicken	0d	Ethyl alcohol	~1	Air cell	Rolled 180° with egg on its side	Control without solvent, solvent control, 8, 16	All at n = 10
10	Chicken	0d	Dimethylsulfoxide	~0.5	Air cell	Rolled 180° with egg on its side	Control without solvent, solvent control, 4, 16, 32	All at n = 10
11	Mallard	4d	0.1N HCl	~1	Albumen[h]	Vertical	Control without solvent Solvent control, 1.6	25 30
12	Mallard	4d	0.1N HCl	8	Albumen	Vertical	Control without solvent, solvent control, 4	All at 27 to 28
13	Mallard	4d	4% chicken ovalbumin dissolved in 0.1N HCl	~1	Albumen	Vertical	Control without solvent Solvent control, 1.6	25 30

Table 1. Continued.

Experiment No.[a]	Species	Age of embryo (days and hours)	Solvent	Volume of solvent (µl/g of egg contents)	Site of injection	Egg position during incubation	Mercury concentrations injected into eggs (µg/g of egg contents on a wet-weight basis)	Sample size for each group listed
14 (part 1)	Mallard	4d	1% chicken ovalbumin dissolved in 0.1N HCl	~4	Albumen	Vertical	Control without solvent Solvent control, 4	30 27 to 28
14 (part 2)	Mallard	4d	1% chicken ovalbumin dissolved in 0.1N HCl	~4	Albumen	Rotated 60° with egg on its side	Solvent control, 4	27 to 28
15 (part 1)	Mallard	4d	68.75% mallard albumen/31.25% water	20	Albumen	Vertical	Control without solvent Solvent control, 4	28 27 to 28
15 (part 2)	Mallard	4d	97.78% mallard albumen/2.22% propylene glycol	20	Albumen	Vertical	Solvent control, 4	27 to 28
16 (part 1)	Mallard	4d	1% methionine in 0.1N HCl	1	Albumen	Vertical	Control without solvent Solvent control, 1.6	25 30
16 (part 2)	Mallard	4d	1% cysteine in 0.1N HCl	1	Albumen	Vertical	Solvent control, 1.6	30
Experiments designed primarily to compare the survival of eggs injected with different volumes of solvent								
17 (part 1)	Mallard	4d	Corn oil	0.5	Air cell	Vertical	Control without solvent Solvent control	28 28
17 (part 2)	Mallard	4d	Corn oil	1	Air cell	Vertical	Solvent control	28
18 (part 1)	Mallard	4d	Corn oil	0	Air cell	Rolled 180° with egg on its side	Control without solvent	20
18 (part 2)	Mallard	4d	Corn oil	0.25	Air cell	Rolled 180° with egg on its side	Solvent control, 1.6	20
18 (part 3)	Mallard	4d	Corn oil	0.5	Air cell	Rolled 180° with egg on its side	Solvent control, 1.6	19 to 20
18 (part 4)	Mallard	4d	Corn oil	1	Air cell	Rolled 180° with egg on its side	Solvent control, 1.6	20
18 (part 5)	Mallard	4d	Corn oil	2	Air cell	Rolled 180° with egg on its side	Solvent control, 1.6	20
19 (part 1)	Double-crested cormorant	4d	Corn oil	0	Air cell	Rolled 180° with egg on its side	Control without solvent	12
19 (part 2)	Double-crested cormorant	4d	Corn oil	0.25	Air cell	Rolled 180° with egg on its side	Solvent control, 1.6	14
19 (part 3)	Double-crested cormorant	4d	Corn oil	0.5	Air cell	Rolled 180° with egg on its side	Solvent control, 1.6	13 to 15
19 (part 4)	Double-crested cormorant	4d	Corn oil	1	Air cell	Rolled 180° with egg on its side	Solvent control, 1.6	14
19 (part 5)	Double-crested cormorant	4d	Corn oil	2	Air cell	Rolled 180° with egg on its side	Solvent control, 1.6	13 to 14

Table 1. Continued.

Experiment No.[a]	Species	Age of embryo (days and hours)	Solvent	Volume of solvent (µl/g of egg contents)	Site of injection	Egg position during incubation	Mercury concentrations injected into eggs (µg/g of egg contents on a wet-weight basis)	Sample size for each group listed
Experiments designed primarily to compare the survival of eggs injected into the air cell versus into the albumen								
20 (part 1)	Chicken	0d	Propylene glycol	~1	Albumen[i]	Rotated 60° with egg on its side	Control without solvent, solvent control, 16	All at n = 10
20 (part 2)	Chicken	0d	Ethyl alcohol	~1	Albumen	Rotated 60° with egg on its side	Solvent control, 16	5
20 (part 3)	Chicken	0d	Dimethylsulfoxide	~1	Albumen	Rotated 60° with egg on its side	Solvent control, 16	5
21 (part 1)	Mallard	4d	Corn oil	~1	Air cell	Vertical	Control without solvent Solvent control, 1.6	25 29 to 30
21 (part 2)	Mallard	4d	Corn oil	~1	Albumen	Vertical	Solvent control, 1.6	30
22 (part 1)	Mallard	4d	Propylene glycol	~0.5	Air cell	Vertical	Control without solvent Solvent control, 4	30 15
22 (part 2)	Mallard	4d	Propylene glycol	~0.5	Albumen	Vertical	Solvent control, 4	15
23	Mallard	4d	12.5% propylene glycol/87.5% water	~4	Albumen	Vertical	Control without solvent Solvent control, 4	30 15
24 (part 1)	Mallard	5d	4% chicken ovalbumin dissolved in 0.1N HCl	~10	Air cell	Vertical	Solvent control, 1, 4, 16	24 to 30
24 (part 2)	Mallard	5d	4% chicken ovalbumin dissolved in 0.1N HCl	~10	Albumen	Vertical	Solvent control, 1, 2, 4, 8, 16	28 to 30
Experiments designed primarily to compare survival of eggs when the injections were made at different embryonic ages								
25	Chicken	0d	Corn oil	1	Air cell	Vertical	Control without solvent Solvent control 0.5, 1, 2, 4, 8, 12, 16	150 117 55 to 59
26	Chicken	0d	Corn oil	~1	Air cell	Rotated 60° with egg on its side	Control without solvent, solvent control, 2, 4, 8, 12, 16	All at 9 to 10
27 (part 1)	Chicken	3d	Corn oil	1	Air cell	Rolled 180° with egg on its side	Control without solvent Solvent control 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2	60 60 29 to 30
27 (part 2)	Chicken	4d	Corn oil	1	Air cell	Rolled 180° with egg on its side	Solvent control 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2	35 20
28	Mallard	0d	Corn oil	1	Air cell	Vertical	Control without solvent Solvent control 0.5, 1, 2, 4, 8, 12, 16	59 60 57 to 60
29 (part 1)	Mallard	−1d[j]	Corn oil	0.5	Air cell	Vertical	Control without solvent, solvent control, 2	All at n = 30
29 (part 2)	Mallard	4d	Corn oil	0.5	Air cell	Vertical	Solvent control, 2	30
30 (part 1)	Mallard	3d	Corn oil	1	Air cell	Rolled 180° with egg on its side	Control without solvent Solvent control, 0.4, 0.8, 1.6, 3.2, 6.4	20 20
30 (part 2)	Mallard	4d	Corn oil	1	Air cell	Rolled 180° with egg on its side	Solvent control, 0.4, 0.8, 1.6, 3.2, 6.4	20

Table 1. Continued.

Experiment No.[a]	Species	Age of embryo (days and hours)	Solvent	Volume of solvent (µl/g of egg contents)	Site of injection	Egg position during incubation	Mercury concentrations injected into eggs (µg/g of egg contents on a wet-weight basis)	Sample size for each group listed
30 (part 3)	Mallard	5d	Corn oil	1	Air cell	Rolled 180° with egg on its side	Solvent control, 0.4, 0.8, 1.6, 3.2, 6.4	18 to 20
31 (part 1)	Mallard	−1d	Corn oil	0.5	Air cell	Vertical	Control without solvent, solvent control, 2, 8	25
31 (part 2)	Mallard	0d	Corn oil	0.5	Air cell	Vertical	Solvent control, 2, 8	25
31 (part 3)	Mallard	1d	Corn oil	0.5	Air cell	Vertical	Solvent control, 2, 8	25
31 (part 4)	Mallard	2d	Corn oil	0.5	Air cell	Vertical	Solvent control, 2, 8	25
31 (part 5)	Mallard	4d	Corn oil	0.5	Air cell	Vertical	Solvent control, 2, 8	24 to 25
32 (part 1)	Double-crested cormorant	3d	Corn oil	1	Air cell	Rolled 180° with egg on its side	Control without solvent Solvent control, 1.6	12 10
32 (part 2)	Double-crested cormorant	4d	Corn oil	1	Air cell	Rolled 180° with egg on its side	Solvent control, 1.6	11
32 (part 3)	Double-crested cormorant	5d	Corn oil	1	Air cell	Rolled 180° with egg on its side	Solvent control, 1.6	11 to 13
33	Mallard	1d, 23h[k]	Propylene glycol	∼1	Air cell	Rolled 180° with egg on its side	Control without solvent Solvent control 1, 2, 4, 8, 16	60 20 18 to 20
34	Mallard	1d, 22h	Propylene glycol	1	Air cell	Rolled 180° with egg on its side	Control without solvent Solvent control 2, 4, 8, 16, 32	30 30 29 to 30
35	Ring-necked pheasant	1d, 18h[l]	Propylene glycol	1	Air cell	Rolled 180° with egg on its side	Control without solvent Solvent control 2, 4, 8, 16, 32	28 21 17 to 27
36	Ring-necked pheasant	2d, 3h[m]	Propylene glycol	1	Air cell	Rolled 180° with egg on its side	Control without solvent Solvent control 1, 2, 4, 8, 16	50 29 10 to 13

[a] Experiment numbers are listed in sequential order through the text.
[b] A 4-day-old mallard embryo resembles a 3-day-old chicken embryo in appearance.
[c] Mallard or chicken eggs that received ∼1 µl of solvent per gram of egg contents actually were injected with a total of 50 µl solvent for mallards or 60 µl for chickens, which, based on the weight of the eggs for these two species, is approximately 1 µl/g egg contents. Likewise, the designations ∼0.5 µl, ∼4 µl, or ∼10 µl were approximately 0.5, 4, or 10 µl/g of egg contents.
[d] Each of the control groups and each group injected with different concentrations of mercury had a sample size of 55 eggs.
[e] Each of the groups had a sample size that varied from 29 to 30 eggs.
[f] A 3-day, 7 hour-old ring-necked pheasant embryo is similar in appearance to a 3-day-old chicken embryo.
[g] The injections were made just prior to the start of incubation.
[h] Unless specified otherwise, injections into the albumen were done through a hole drilled in the apex of the egg.
[i] Injections into the albumen in this experiment were done through a hole drilled in the cap of the egg and a needle was inserted through the inner shell membrane of the egg.
[j] Eggs designated as being injected after −1 day of incubation were injected and then allowed to sit at room temperature for one day prior to being placed in the incubator.
[k] A 1-day, 23 hour-old mallard embryo is similar in appearance to a 1.5-day-old chicken embryo.
[l] A 1-day, 18 hour-old ring-necked pheasant embryo is similar in appearance to a 1.5-day-old chicken embryo.
[m] A 2-day, 3 hour-old ring-necked pheasant embryo is similar to a 2-day-old chicken embryo.

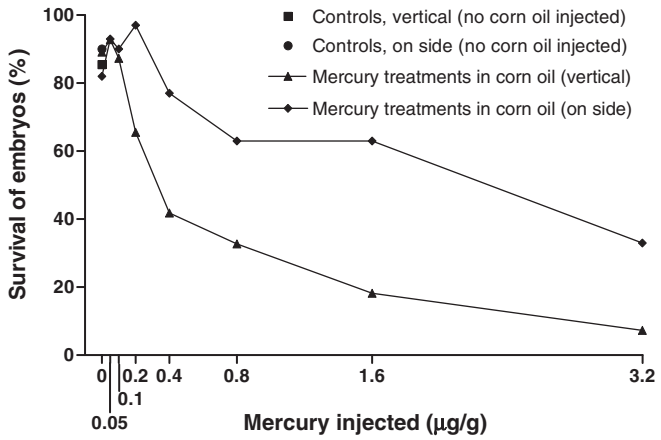

Fig. 1. Survival of mallard embryos through 90% of incubation when the eggs were incubated in a vertical position versus lying on their sides

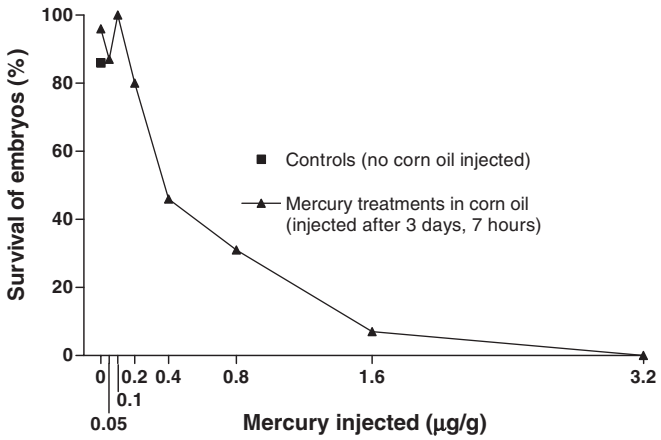

Fig. 2. Survival of ring-necked pheasant embryos through 90% of incubation when the eggs were lying on their sides

with 1.6 μg/g mercury, 40% of those incubated on their sides survived, which was significantly greater than the 6.7% survival of those incubated vertically. Comparison of the results from experiments 3 and 4 demonstrated that although survival of controls was good under all conditions, methylmercury was more toxic when the injected eggs were incubated in a vertical position.

In later experiments, we adopted the practice of incubating eggs on their sides and rolling them 180°/h because of the exaggerated toxicity of methylmercury when eggs were incubated vertically and also because wild bird eggs fared better when incubated on their sides.

Choice of a Solvent: Corn Oil

Experiment 2. In Figure 1, the data for eggs incubated on their sides show that corn oil is a good solvent when injected at the morphologic equivalent (based on degree of embryonic development and spread of blood vessels over the surface of the yolk) of a 3-day-old chicken embryo. Control eggs injected with corn oil survived approximately as well as uninjected controls, and a clear dose-response curve was evident.

Experiment 5. Groups of ring-necked pheasant eggs were injected after 3 days and 7 hours of incubation, at which time the pheasant embryos resembled 3-day-old chicken embryos. Controls injected with corn oil survived just as well as uninjected controls, and, as was the case in experiment 2 with mallards, mercury produced a clear dose-response curve (Fig. 2).

Choice of a Solvent: Propylene Glycol

Experiment 6. Chicken eggs were injected with 60 μl methylmercury dissolved in propylene glycol. For chicken eggs, 60 μl is approximately 1 μl/g egg contents. The injections were done just before the start of incubation (“day 0” in Table 1). We tried to dissolve enough methylmercury in the propylene glycol to produce a concentration of 24 μg/g mercury, but the methylmercury would not stay in solution. All 10 of the uninjected control eggs and the 10 controls injected with pure propylene glycol survived. With the 4-μg/g mercury dose, 9 of 10 eggs survived, and with the 16-μg/g dose, 10 of 10 eggs survived. We concluded that propylene glycol had no toxicity when injected just before the start of incubation, but no mortality occurred with the high mercury doses.

In addition to the toxicity study, we conducted a concurrent study in which we made a 3% solution of trypan blue dye dissolved in propylene glycol and injected the solution into the air cell of 10 extra chicken eggs. After 3 hours, the blue dye had moved into the albumen and was concentrated under the inner shell membrane. When a sample of eggs was opened after 3 days, the entire albumen was colored a light blue. No penetration of the dye into the yolk was observed, although the membrane around the yolk took up the dye.

Choice of a Solvent: Soybean Oil

Experiment 7. In this study we compared the toxicity of methylmercury dissolved in soybean oil versus corn oil. Survival was similar for the corn oil controls (90%), soybean controls (86.7%), and uninjected controls (86.7%). Survival was greatly decreased in the eggs injected with 2 μg/g mercury, whether dissolved in corn oil (6.7%) or soybean oil (13.3%).

Choice of a Solvent: Crisco

Experiment 8. The Crisco had to be heated and converted to a liquid to dissolve the methylmercury. Percent survival of uninjected mallard eggs was 89.3% compared with 71.4% for the controls injected with 0.5 μl pure melted Crisco/g egg contents and 78.6% for controls injected with 1 μl Crisco/g egg contents. Only 7.1% of the eggs injected with 4 μg/g mercury in 0.5 μl Crisco/g egg contents survived through 90% of incubation compared with 0% for eggs injected with 4 μg/g mercury in 1 μl Crisco/g egg contents. We noted that, after being injected, the Crisco returned to a solid drop on top of the inner shell membrane. This characteristic made it unsatisfactory as a solvent.

Choice of a Solvent: Ethyl Alcohol

Experiment 9. Chicken eggs were injected with solutions of methylmercury dissolved in anhydrous ethyl alcohol. All 10 of the uninjected control eggs and the controls injected with pure ethyl alcohol survived. In both the 8 and 16 μg/g mercury doses, 7 of the 10

eggs survived. We concluded that ethyl alcohol had no toxicity when injected just before the start of incubation, but only a modest amount of mercury-induced mortality occurred, perhaps owing to the fact that, at least at room temperature, some of the methylmercury at 8 and 16 μg/g began to precipitate out. In addition to the toxicity study, we conducted a concurrent study in which we made a 3% solution of Sudan IV dye dissolved in ethyl alcohol and injected the solution into the air cell of 10 extra chicken eggs. The dye-stained ethyl alcohol immediately began to pass through the inner shell membrane.

Choice of a Solvent: Dimethylsulfoxide

Experiment 10. Chicken eggs were injected with methylmercury dissolved in dimethylsulfoxide (DMSO). All 10 of the uninjected control eggs survived compared with 8 of 10 for the controls injected with pure DMSO. Nine of 10 eggs injected with 4 μg/g mercury in DMSO survived, as did 9 of 10 injected with 16 μg/g mercury and 10 of 10 injected with 32 μg/g mercury.

In conjunction with the toxicity study, we prepared a 3% solution of trypan blue dye dissolved in DMSO and injected the solution into the air cell of 10 extra chicken eggs. Within 3 hours the blue dye showed up in the albumen of the eggs. After 3 days, the dye was uniformly distributed in the albumen of the egg, better even than dye dissolved in propylene glycol. There was no evidence that the blue dye had penetrated into the yolk. We concluded that although methylmercury dissolves easily in DMSO and the DMSO itself had little if any toxicity when injected just before the start of incubation, the disadvantage of DMSO was that even high doses of methylmercury exhibited no toxicity when dissolved in this solvent.

Choice of a Solvent: HCl Solutions

Experiment 11. Methylmercury is not very soluble in pure water. Consequently, we made a 0.1N HCl solution in water to dissolve the methylmercury. The injections were made directly into the albumen of mallard eggs through a hole made in the apex. Survival was 100% for the controls injected with pure 0.1N HCl, which was not significantly different from the 88% survival for the set of uninjected controls. Only 13.3% of the eggs injected with 1.6 μg/g mercury in the 0.1N HCl solution survived. The weak HCl solution seemed to be safe for embryos and produced a high level of mortality when mixed with methylmercury. Injections directly into the albumen of an egg, however, are more difficult to do. Although not the case in this experiment, we discuss later how high mortality often occurs in controls injected with various solvents through the apex of the egg.

Experiment 12. We first made a small hole in the cap end of mallard eggs, and then drilled another hole in the apex end. The hole in the cap allowed air to escape from the air cell so the inner shell membrane could sag down, creating more space under the hole in the apex to inject the large volume (469 μl) of solvent into the albumen of the egg. Only 33.3% of the controls injected with pure 0.1N HCl survived compared with 89.3% for the uninjected controls. Survival of eggs injected with 4 μg/g mercury was 7.1%. The low survival of the injected controls differs from the results in the previous experiment, in which survival was 100%. In other experiments, where we carefully examined the eggs for air bubbles, we have often observed these bubbles when the injection was into the albumen. It is possible that harmful air bubbles were created in the second experiment, where we first made a hole in the cap end of the egg to allow for a sagging of the inner shell membrane.

Choice of a Solvent: Purified Chicken Ovalbumin and Mallard Albumen Solutions

Experiment 13. Purified chicken ovalbumin contains sulfhydryl-containing proteins, and methylmercury attaches to sulfhydryl groups (Simpson *et al.* 1973; Nishimura and Urakawa 1976). Therefore, we thought a solution of methylmercury dissolved in chicken ovalbumin might be a more natural way to present the mercury in the egg. A weak HCl solution was required to dissolve the methylmercury, after which the ovalbumin was added. Groups of mallard eggs were injected with 4% chicken ovalbumin dissolved in a 0.1N HCL solution. Only 60% of the controls injected with albumin survived, which was significantly lower than the 88% survival for the set of uninjected controls. Of the eggs injected with 1.6 μg/g mercury in the 4% albumin solution, 66.7% survived, suggesting that the albumin solution had prevented methylmercury toxicity.

Experiment 14. Mallard eggs were injected into the albumen with a solution of 1% ovalbumin in 0.1N HCl and were incubated with the eggs held in either a vertical position or on their sides. The most noticeable result was that the survival of the two sets of control eggs (set vertically or on their sides) was only 53% and 47%, respectively. In contrast, the uninjected controls had 100% survival. Survival for the mercury-injected eggs was 13% for the eggs set vertically and 27% for the eggs set on their sides. Again, we observed that it is difficult to inject eggs through a hole drilled in the apex without causing mortality.

Experiment 15. Mallard eggs were injected into the albumen with methylmercury in two different solutions of mallard albumen. We first made a hole in the cap and then another in the apex, allowing the inner shell membrane to sag down. In one part of the study, the methylmercury was first dissolved in distilled, deionized water and then mixed with mallard albumen (collected from extra mallard eggs). Only 42.9% of the injected controls survived through 90% of incubation compared with 89.3% of the uninjected controls. Of the eggs injected with 4 μg/g mercury, 21.4% survived.

In another part of the study we first dissolved the methylmercury in propylene glycol and then added mallard albumen. Only 66.7% of the controls injected with the pure propylene glycol–albumen solution survived. This albumen solution conferred protection against methylmercury poisoning: 63.0% of the eggs injected with 4 μg/g mercury survived. Our use of mallard albumen instead of purified chicken ovalbumin did not eliminate the harmful effects associated with the use of albumen solutions to act as a carrier for methylmercury. Either the albumen itself was toxic or the introduction of air bubbles by injecting solutions through the apex caused mortality.

Choice of a Solvent: Amino Acid Solutions

Experiment 16. We tested solutions containing methionine (which contains a sulfur–methyl bond) or cysteine (which contains a sulfur–hydrogen bond) because these two amino acids, like the proteins in albumen, attract methylmercury to the sulfur atom (Simpson *et al.* 1973). Mallard eggs were injected directly into the albumen with either 1% methionine in 0.1N HCl solution or 1% cysteine in 0.1N HCl solution. Survival of neither the methionine controls (96.7%) nor the cysteine controls (93.3%) significantly differed from survival of the uninjected controls (88%). The addition of 1.6 μg/g mercury to the methionine and cysteine solutions resulted in 23.3% and 26.7% survival, respectively.

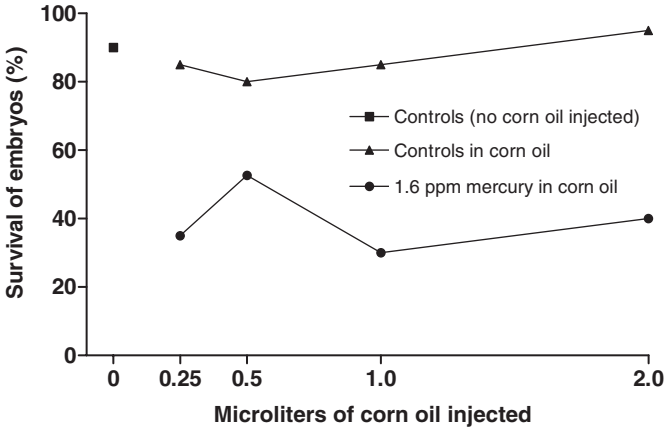

Fig. 3. Survival of mallard embryos through 90% of incubation when the eggs were injected with no corn oil, pure corn oil, or 1.6 μg/g mercury in 0.25, 0.5, 1, or 2 μl corn oil/g egg contents

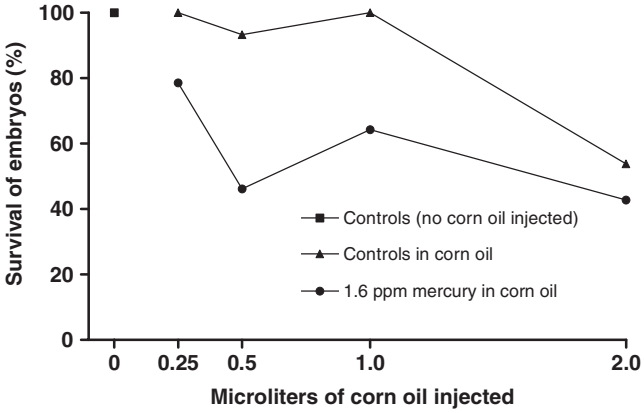

Fig. 4. Survival of double-crested cormorant embryos through 90% of incubation when the eggs were injected with no corn oil, pure corn oil, or 1.6 μg/g mercury in 0.25, 0.5, 1, or 2 μl corn oil/g egg contents

We chose to use corn oil in our final protocol, although some of the other solvents had their own advantages, and we may have been able to select another with satisfactory results. Methylmercury chloride dissolved readily in propylene glycol, and because propylene glycol is soluble in water, it had an advantage over corn oil in that it mixed evenly into the albumen of the egg. However, propylene glycol was toxic to embryos at the 3-day chicken stage, whereas corn oil was not. Propylene glycol could be safely injected into bird eggs before the start of incubation, but the resulting toxicity of methylmercury was much less than when the mercury is deposited in the egg by the mother. At the 3-day chicken stage, the toxicity of injected methylmercury in corn oil was closer to that of methylmercury naturally deposited in eggs by the mother than when many of the other solvents were used.

Volume of Solvent

Experiment 17. Groups of mallard eggs were injected with either 0.5 or 1 μl corn oil. Survival of the uninjected controls was 89.3% compared with 92.9% for the controls injected with 0.5 μl of corn oil/g egg contents and 89.3% for the controls injected with 1 μl corn oil/g egg contents. These differences were not statistically significant.

Experiment 18. Mallard eggs were injected with no corn oil or with 0.25, 0.5, 1, or 2 μl corn oil/g egg contents. Among the control groups, there were no significant differences in embryo survival between the eggs that were not injected with any corn oil and those injected with various volumes of corn oil (Fig. 3). The dose of 1.6 μg/g mercury caused an expected decrease in embryo survival compared with controls, but there were no significant differences in survival between eggs injected with 1.6 μg/g mercury in 0.25 μl corn oil/g egg contents and those injected with the mercury in 0.5, 1, or 2 μl of corn oil/g egg contents.

Experiment 19. Groups of double-crested cormorant eggs were injected with no corn oil or with 0.25, 0.5, 1, or 2 μl corn oil/g egg contents. Unlike the mallard study above, in which the volume of injected corn oil had no significant effect on embryo survival, embryo survival of cormorant eggs was significantly less when control eggs were injected with 2 μl corn oil/g egg contents than when injected with no corn oil (Fig. 4). Survival of eggs injected with 0.25, 0.5, and 1 μl/g egg contents did not differ from that of the controls receiving no corn oil. As was the case in the mallard experiment, 1.6 μg/g mercury caused a decrease in survival of cormorant eggs compared with controls. There also was a suggestion of decreased survival with higher amounts of corn oil, but none of these differences was significant ($p = 0.12$ for 0.25 μl versus 0.5 μl, and $p = 0.12$ for 0.25 μl versus 2 μl; Fisher's exact probability tests).

For our final protocol, we elected to inject 1 μl corn oil/g egg contents. There was evidence from the cormorant experiment that more than 1 μl could be toxic. After settling on 1 μl corn oil, we began studies in which we injected graded doses of methylmercury into the eggs of many wild species of birds (unpublished data). With the eggs of some species of wild birds, where we had enough eggs to assign one group to be uninjected controls, we did observe a greater mortality of embryos in eggs injected with 1 μl pure corn oil/g egg contents than in the uninjected eggs. In hindsight, it might have been better to use 0.5 μl corn oil. The advantage of using more corn oil is that a greater volume of solvent is available to carry the methylmercury across the inner shell membrane and distribute it evenly throughout the albumen of the egg.

Site of Injection

In cases where it is not feasible to feed the mother bird methylmercury and have her deposit methylmercury into her eggs, there are four other ways one can get methylmercury into eggs: (1) methylmercury can be dissolved in a solvent and spread on the surface of an egg, or the methylmercury can be dissolved in a solvent and injected into the (2) air cell, (3) albumen, or (4) yolk. Although almost half of the methylmercury dissolved in a mixture of aliphatic hydrocarbons and spread on the surface of the egg passed into the egg within several days of treatment (Hoffman and Moore 1979), we ruled out application to the surface of the shell because it is most removed from the way methylmercury is naturally deposited in the egg by the female bird.

Because methylmercury is found mostly in the albumen of the egg with much less in the yolk (Nishimura and Urakawa 1976; Sell *et al.* 1974; Tejning 1967), we ruled out yolk injections. That left injections into the air cell or directly into the albumen. The following experiments were conducted to compare the results and utility of air cell versus albumen injections.

Experiment 20. Chicken eggs were injected into the albumen with propylene glycol. Unlike most other studies in which we injected a solvent directly into the albumen through a hole drilled in the apex of the egg, the propylene glycol in this experiment was injected into the albumen by drilling a hole in the cap of the egg and then inserting a needle into the air cell and then through the inner shell membrane. All 10 of the uninjected controls survived, 9 of 10 propylene glycol controls survived, and 8 of 10 of the eggs injected with 16 μg/g mercury survived through 90% of incubation.

In addition to propylene glycol, sets of eggs were injected with ethyl alcohol or DMSO. These injections also were made into the albumen through the inner shell membrane of the egg. Four of 5 alcohol controls survived, as did 4 of 5 eggs injected with 16 μg/g mercury in alcohol, 5 of 5 DMSO controls, and 5 of 5 injected with 16 μg/g mercury in DMSO.

Although the sample sizes were small, the results from these three different solvents were encouraging in that they clearly showed that no mortality was caused by injecting into the albumen through the cap versus the apex of the egg. We did not check the eggs for air bubbles, but this method of albumen injection probably does not create air bubbles because a hole is never made through the shell directly into the albumen. What was not encouraging was the observation that a dose of 16 μg/g mercury caused no mortality. This failure of even a very high concentration of injected mercury to cause embryo mortality is consistent with the results of other studies in which we injected the eggs just before the start of incubation.

Experiment 21. Mallard eggs were injected either into the air cell or directly into the albumen of the egg through a hole in the apex. Survival of control embryos was 88% for the uninjected controls, 89.7% for controls injected into the air cell, and 90% for controls injected into the albumen. Survival of the group injected with 1.6 μg/g mercury in corn oil into the air cell was 6.67%, which was not significantly different from the 20% for eggs injected with 1.6 μg/g mercury into the albumen.

Experiment 22. Mallard eggs were injected with propylene glycol either into the air cell or the albumen. Embryo survival was 100% for the uninjected controls, which was significantly better than the value of 73.3% for air cell-injected controls and the value of 40% for the albumen-injected controls. As will be discussed further in the next section, the injection of pure propylene glycol after incubation is underway can be harmful to the embryo.

Survival of the eggs treated with 4 μg/g mercury was 0% when the air cell was the site of the injection versus 26.7% when injection was directly into the albumen. This difference was nearly significant ($p = 0.10$), suggesting that methylmercury in propylene glycol may be less toxic when injected into the albumen, although albumen injections run the risk of creating air bubbles.

Experiment 23. Mallard eggs were injected into the albumen with 200 μl of a solution of 12.5% propylene glycol and 87.5% deionized water. A separate hole was made in the cap of the egg to allow the inner shell membrane to sag down to accommodate the 200 μl of solution. Only 40% of the injected controls survived, which was significantly less than the 100% survival of the uninjected controls. The poor survival of the albumen-injected controls was likely related to the toxicity of propylene glycol when injected at 4 days of age plus the possible formation of air bubbles in the egg. Survival of the group injected with 4 μg/g mercury was 26.7%.

Experiment 24. Mallard eggs were injected directly into the albumen or air cell with 500 μl of a solution of 4% purified chicken ovalbumin in 0.1N HCl. Mixing 4% ovalbumin in 0.1N HCl required filtering the solution to remove some denatured protein that precipitated out of the solution. Although we first made a small hole in the cap end of the egg and then another hole in the apex, which allowed the inner shell membrane to sag down, in some eggs some of the thick albumen from the egg bubbled up out of the hole in the apex as we were injecting the 500 μl of solution. We discarded these eggs and replaced them with extra eggs that we had successfully injected. The air cell was able to accommodate the 500 μl solution. Embryo survival through 90% of incubation was 50%, 46.7%, 16.7%, 10%, 0%, and 10% for the eggs injected directly into the albumen with 0, 1, 2, 4, 8, and 16 μg/g mercury, respectively. When the mercury solutions were injected into the air cell, the survival of embryos was 37.5%, 34.5%, 16.7%, and 0% for the eggs injected with 0, 1, 4, or 16 μg/g mercury. With both sites of injection, there was a dose-response pattern from the mercury. However, control mortality was high, suggesting that the ovalbumin–HCl mixture itself was harmful, regardless of injection site. An additional problem with the injections into the albumen was the difficulty of making enough room inside the apex end of the egg for 500 μl of solution.

Because most of the methylmercury in an egg is bound to the protein fraction in egg albumen (Nishimura and Urakawa 1976), injecting methylmercury directly into the albumen of an egg might be considered the most appropriate route. We did try this route with a number of solvents, including solutions containing purified hen's albumin. However, we encountered problems, including the creation of air bubbles when a hole was drilled through the apex of an egg. To drill through the apex without egg white leaking out, we turned the egg upside down. As soon as a hole is made through the shell at the apex, the weight of the egg contents pushes down on the air cell at the opposite end of the egg, causing the inner shell membrane to sag, and air is pulled into the egg. This air pocket created in the apex makes it possible to inject a solution into the egg, but unless all the air is forced out by the injected solution, air bubbles will remain after the hole is sealed. These air bubbles drift about in the egg, always floating to the upper most part of the egg, which is where the embryo settles, and the bubbles often cause embryo mortality. In one chicken experiment, embryo survival was good when we drilled a hole in the cap end of the egg and injected into the albumen through a hole made in the inner shell membrane. However, the amount of solvent that can be injected into the albumen through a hole in the inner shell membrane is limited.

In addition to the problems created by air bubbles, injecting a solution directly into the albumen requires more sterile conditions than when injecting into the air cell, where the inner shell membrane provides protection against invasion by bacteria. We believe it would be possible, with care, to successfully inject solutions containing mercury directly into the egg albumen, but it would be a more time-consuming and difficult procedure than injecting into the air cell. Because the majority of the dissolved methylmercury injected into the air cell passes through the inner shell membrane and into the albumen anyway, we concluded that air cell injections are a fast and safe way to get good dose-response results.

Embryonic Age When Eggs Are Injected: Corn Oil

Experiment 25. This experiment with chicken eggs was the first in which we discovered that corn oil is toxic to embryos when it is injected into the air cell of the egg before the start of incubation (Fig. 5). Methylmercury, even at the highest concentrations we used, had no additional harmful effect on embryo survival. For reasons we do not know, the toxicity of methylmercury is much decreased when injected before or very early in incubation. This holds true with other solvents as well.

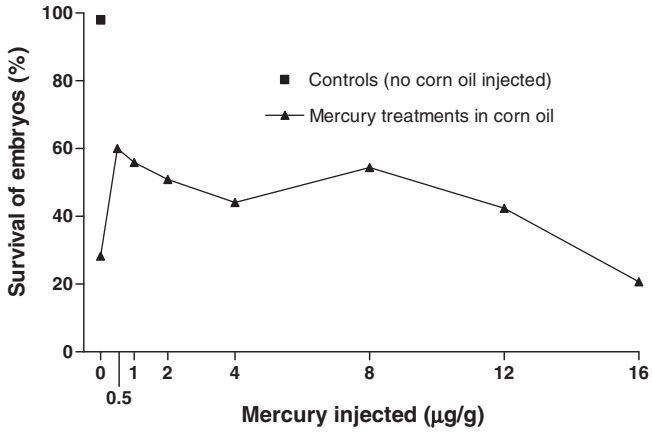

Fig. 5. Survival of chicken embryos through 90% of incubation when the eggs were injected just before the start of incubation with no corn oil or with various doses of mercury dissolved in 1 µl corn oil/g egg contents

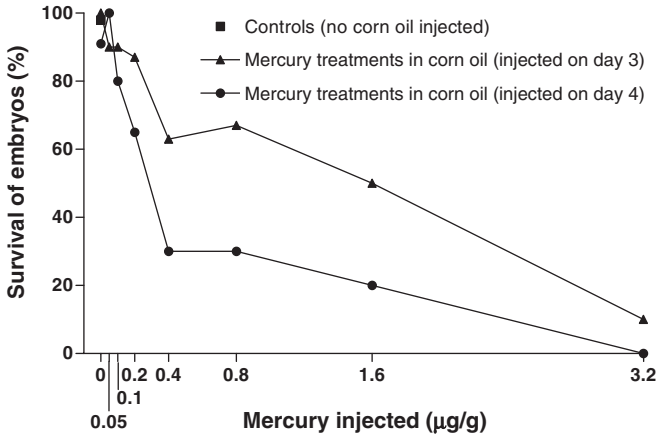

Fig. 6. Survival of chicken embryos through 90% of incubation when the eggs were injected after 3 or 4 days of incubation with no corn oil or with various doses of mercury dissolved in 1 µl corn oil/g egg contents

Experiment 26. Chicken eggs were injected into the air cell with corn oil at day 0. All 10 of the uninjected eggs survived through 90% of incubation compared with only 4 of 10 for the controls receiving pure corn oil. Survival of the mercury-dosed groups was 7 of 9 for the 2-µg/g group, 8 of 10 for the 4-µg/g group, 4 of 10 for the 8-µg/g group, 6 of 10 for the 12-µg/g group, and 4 of 10 for 16-µg/g group. Not only did the pure corn oil cause mortality, but the survival of eggs in the mercury-dosed groups was unrelated to dose and was in some cases better than in the corn oil controls.

As part of this toxicity study we examined the transport of dye dissolved in corn oil out of the air cell and into the albumen of the egg. Ten extra chicken eggs were injected into the air cell with 120 µl of solution of 3% Sudan IV dye dissolved in corn oil. After 3 days, the inner shell membrane was stained from the air cell down to the opposite end of the egg, and the albumen was lightly stained.

Experiment 27. In other experiments, we had determined that corn oil was not embryotoxic when injected at 3 days of embryonic age in the chicken. We wanted to learn what effect corn oil might have on slightly older embryos, so we injected chicken eggs at both 3 and 4 days of age. Embryo mortality was greater for the 0.4, 0.8, and 1.6 µg/g mercury doses when eggs were injected after 4 days than when injected after 3 days of incubation (Fig. 6). Embryo survival among controls injected with pure corn oil at either day 3 or 4 of incubation did not differ from survival of the set of control eggs that was not injected with corn oil.

Experiment 28. Mallard eggs were injected just before the start of incubation, and we observed that pure corn oil, without any added mercury, resulted in severely decreased embryo survival (Fig. 7), confirming the results from other experiments. All of the mercury-treated eggs survived approximately as well as the controls injected with corn oil.

Experiment 29. One group of mallard eggs was injected with corn oil and held at room temperature for 1 day before incubation was begun, whereas another group was injected after 4 days of incubation. Survival of embryos was 86.7% for the uninjected controls, 73.6% for controls injected with pure corn oil 1 day before incubation, 93.3% for controls injected after 4 days of incubation, 86.7% for eggs injected with 2 µg/g mercury 1 day before the start of incubation, and 0% for the group of eggs injected with 2 µg/g mercury after 4 days of incubation. The toxicity of the methylmercury was greatly decreased by injecting before incubation, whereas injecting before the start of incubation increased the toxicity of the pure corn oil.

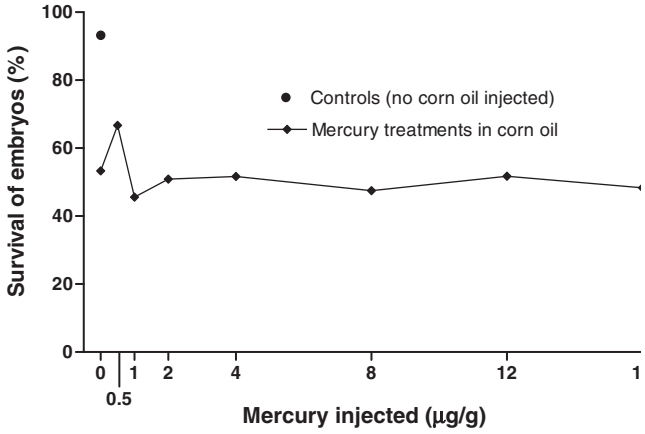

Fig. 7. Survival of mallard embryos through 90% of incubation when the eggs were injected just before the start of incubation with no corn oil or with various doses of mercury dissolved in 1 µl corn oil/g egg contents

Experiment 30. Groups of mallard eggs were injected with corn oil after 3, 4, or 5 days of incubation. Survival of the uninjected controls was 85% compared with 80%, 80%, and 100% for eggs injected with pure corn oil after 3, 4, or 5 days of incubation, respectively (Fig. 8). Mortality of mercury-injected eggs increased as the dose of mercury increased. Among the groups of eggs injected with 6.4 µg/g mercury, embryo survival was significantly less when the eggs were injected on day 4 versus day 5 (Fisher's exact test; $p = 0.04$). The difference in embryo survival between eggs injected with 6.4 µg/g mercury on day 3 versus day 4 was marginally significant ($p = 0.10$). Embryo survival when eggs were injected with 6.4 µg/g mercury on day 3 was nearly the same as when eggs were injected on day 5. We concluded from this experiment that injecting within a day of the optimum of a 4-day-old mallard had little effect on embryo survival.

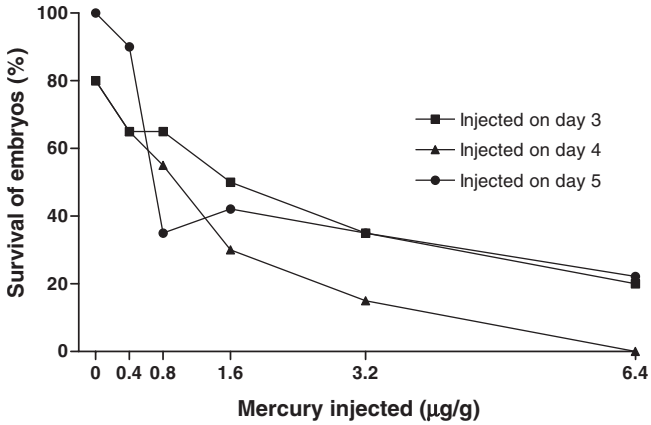

Fig. 8. Survival of mallard embryos through 90% of incubation when the eggs were injected after 3, 4, or 5 days of incubation with no corn oil or with various doses of mercury dissolved in 1 µl corn oil/g egg contents

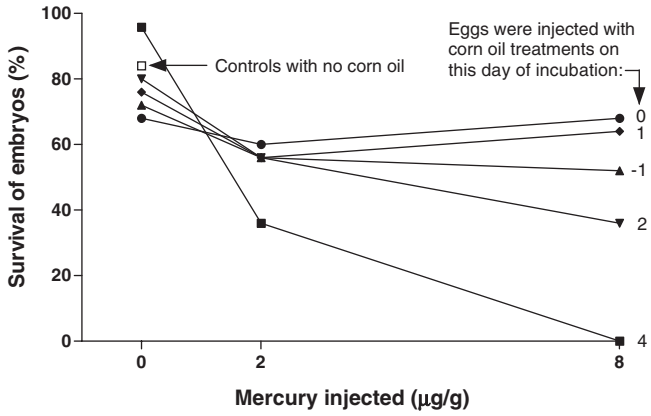

Fig. 9. Survival of mallard embryos through 90% of incubation when the eggs were injected with no corn oil or with 0, 2, or 8 µg/g mercury dissolved in 0.5 µl corn oil/g egg contents and when the injections were made either 1 day before the start of incubation, just before the start of incubation, or 1, 2, or 4 days after the start of incubation

Experiment 31. Mallard eggs were injected with corn oil either 1 day before the start of incubation, just before the start, or after 1, 2, or 4 days of incubation. Although none of the groups of controls injected before day 4 of incubation survived as well as the uninjected controls, none of the differences were statistically significant (Fig. 9). Some of our other experiments had shown a toxicity of pure corn oil when eggs were injected in the first 1 or 2 days of incubation, but perhaps the lower injection volume of only 0.5 µl/g egg contents we used this time was not as toxic. Consistent with what we observed in other experiments, the toxicity of methylmercury decreased when mallard eggs were injected earlier than day 4 of incubation. In the current experiment, when eggs were injected with 8 µg/g mercury, embryo survival was significantly lower when the injection was done after 2 days of incubation than when done immediately before the start of incubation (day 0), and survival of eggs injected with 8 µg/g mercury on day 4 of incubation was significantly lower than for any other day of injection.

Experiment 32. Double-crested cormorant eggs were injected with corn oil after 3, 4, and 5 days of incubation. Neither the injection of 1.6 µg/g mercury into the egg nor the age of the embryo when the egg was injected had a significant effect on embryo survival. Survival of the controls injected with pure corn oil at 3, 4, or 5 days of age was 100%, 82%, and 92%, respectively, and survival of the uninjected eggs was 100%. Survival of eggs injected with 1.6 µg/g mercury at 3, 4, or 5 days of age was 90%, 73%, and 83%, respectively.

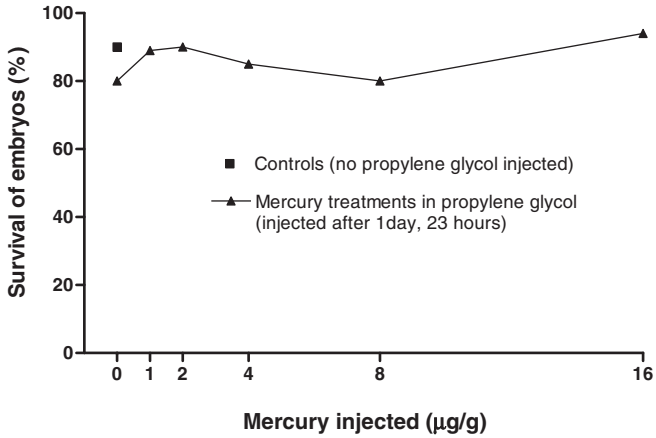

Fig. 10. Survival of mallard embryos through 90% of incubation when the eggs were injected after 1 day, 23 hours of incubation with no propylene glycol or with various doses of mercury dissolved in 55 µl propylene glycol/egg

Embryonic Age When Eggs Are Injected: Propylene Glycol

In experiments discussed previously, we discovered that propylene glycol is embryotoxic when injected at the equivalent of a 3-day-old chicken embryo, but is safe when injected before the start of incubation. The following experiments were designed to determine if propylene glycol would remain safe if injected at stages equivalent to a 1.5- or 2-day-old chicken embryo.

Experiment 33. Mallard eggs were injected with propylene glycol after 1 day, 23 hours of incubation (which is equal to approximately 1.5 chicken days). There was no significant difference in survival between the solvent controls and uninjected controls, showing, at least in this study, that propylene glycol was not toxic to embryos at the 1.5-day chicken stage (Fig. 10). However, the groups injected with increasing concentrations of mercury survived as well as the controls, suggesting that merely increasing the age of the embryos at the time of injection from 0 days of age, as we had used in other experiments, to the equivalent of approximately 1.5 chicken days did not enhance the toxicity of the methylmercury.

Experiment 34. Mallard eggs were injected with propylene glycol after 1 day, 22 hours of incubation (the exact equivalent of a 1.5-day-old chicken embryo). There was no significant difference in the survival of controls injected with pure propylene glycol and controls that were not injected (Fig. 11). Among the mercury-treated groups, only small additional decreases in embryo survival occurred as the mercury concentration in eggs doubled from one dose to the next. These findings support those of the previous experiment.

Experiment 35. Ring-necked pheasant eggs were injected with propylene glycol after 1 day, 18 hours of incubation, which is equivalent to approximately 1.5 days for a chicken embryo. In the

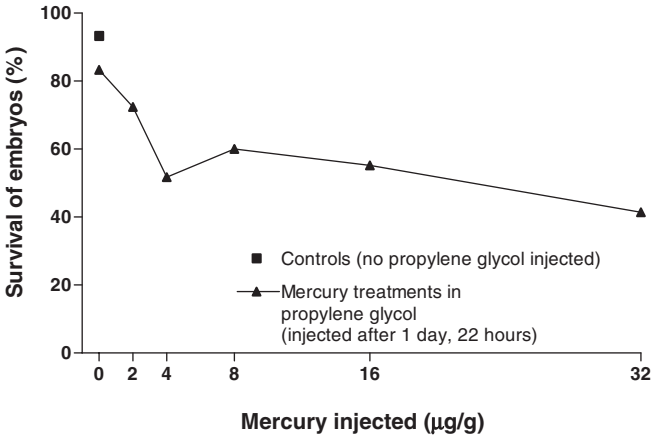

Fig. 11. Survival of mallard embryos through 90% of incubation when the eggs were injected after 1 day, 22 hours of incubation with no propylene glycol or with various doses of mercury dissolved in 1 µl propylene glycol/g egg contents

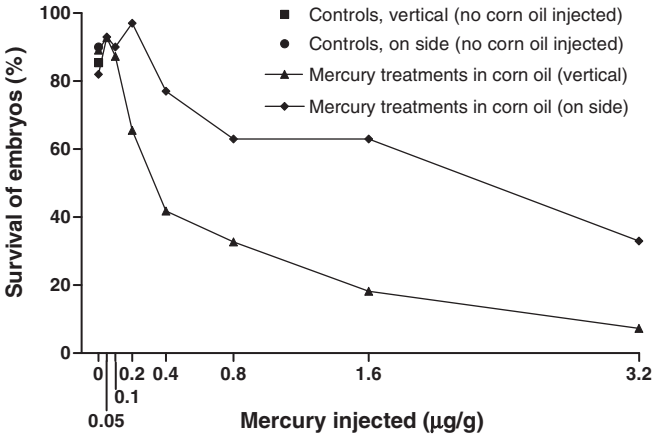

Fig. 12. Survival of ring-necked pheasant embryos through 90% of incubation when the eggs were injected after 2 days, 3 hours of incubation with no propylene glycol or with various doses of mercury dissolved in 1 µl propylene glycol/g egg contents

uninjected controls, 68% survived through 90% of incubation, but only 38% of the controls injected with pure propylene glycol survived. As in other experiments, much higher concentrations of mercury dissolved in propylene glycol were required to cause mortality than when the mercury was dissolved in corn oil and injected at the equivalent of 3 chicken days. Embryo survival was 37%, 35%, 36%, 24%, and 0% in eggs injected with 2, 4, 8, 16, and 32 µg/g mercury in propylene glycol, respectively. We also encountered a problem with the 16- and 32-µg/g mercury solutions: Methylmercury eventually began to precipitate out of the propylene glycol, even in a 40°C solution. In this experiment, we returned the eggs to the incubator immediately after the injection hole had been sealed and held the eggs in a vertical position for the remainder of the 30-minute period.

Experiment 36. Ring-necked pheasant eggs were injected with propylene glycol after 2 days, 3 hours of incubation, at which time the embryos were at a stage equivalent to approximately a 2-day-old chicken embryo. The pure propylene glycol proved toxic to embryos (Fig. 12). Mercury-induced mortality above that experienced by the eggs injected with pure propylene glycol did not occur until the mercury concentrations in eggs reached 8 and 16 µg/g.

Pheasant embryos may be more sensitive to air cell injections of propylene glycol than are mallard embryos when eggs are injected at the embryologic equivalent of a 1.5-day-old or 2-day-old chicken egg. As discussed earlier, injections of propylene glycol made before the start of incubation are not toxic, but neither is the injected methylmercury very toxic when injected then. Even at 1.5 or 2 chicken days, methylmercury does not exhibit enough toxicity to make propylene glycol a suitable solvent.

If one's only goal with egg injections were to mimic the way the mother deposits mercury in her eggs, one would inject eggs just before the time they were placed in an incubator because this would allow for the methylmercury to be in the eggs for the entire incubation period. However, we decided against this approach for a number of reasons. First, without opening an egg it is impossible to determine whether it is fertile. For this reason, sample sizes can become smaller and unequal owing to infertility of eggs. A related problem is that some eggs die within the first 2 or 3 days of incubation from unknown causes. This happens even in groups of control eggs that are not injected with a solvent. It is impossible to distinguish between this natural mortality and early mortality caused by methylmercury. Although infertility and early mortality can be controlled for by including solvent controls in a study, we encountered bigger practical problems related to (1) the toxicity of our most promising solvent, corn oil, when injected before the start of incubation and (2) the lack of toxicity of methylmercury when injected in propylene glycol before the start of incubation.

We decided to standardize our injection protocol so that the injections of the eggs of all species occurred when the embryos reached the developmental equivalent of a 3-day-old chicken embryo. Even when injected a full day before or after the optimum 3-day-old chicken embryo stage, our findings with chicken, mallard, and cormorant eggs suggested that pure corn oil induces minimal toxicity to embryos. Although the toxicity of the methylmercury can vary some when eggs are injected a full day away from the optimum 3-day-old chicken stage, when injecting wild bird eggs one is likely to be off in the aging process by no more than a few hours from a 3-day-old chicken, and consequently the toxicity results are likely to change very little.

Other Procedures Related to Developing the Egg Injection Protocol

Drilling the Hole in the Shell

We used a Dremel rotary tool (Dremel, Racine, WI) to drill the hole in the shell. First we swabbed the Dremel bit and the cap or apex of the egg with an alcohol swab, but other disinfectants would work. Depending on the size of the egg, we drilled holes that varied in size from 1/32 to 3/32 of an inch. The main reason for using smaller holes with the small eggs is to prevent shattering the shell with a big bit and to avoid striking the inner shell membrane. Others have used a tack or pin (Sanderson and Bellward 1995; Powell *et al.* 1998) or a dentist's drill (Brunström and Örberg 1982) to make holes in the eggshell. A small amount of dust or chips from the drilled shell falls into the hole and onto the inner shell membrane when the hole is drilled, but they did not seem to cause any problems.

Injecting the Solvent into the Egg

We used a repeating pipettor for most of our injections. This type of pipettor allows one to inject many eggs without having to reload the pipettor. We discovered that the best way to inject the solvent from the

pipettor into the air cell was to use the standard pipettor tips; with these tips, the solvent does not form a drop that clings to the tip. To calculate the number of microliters of solvent to inject, we first weighed all of the eggs in the study to arrive at a mean whole egg weight. Because we based our concentrations of injected methylmercury on the weight of the contents of the egg, we subtracted an estimate of the eggshell weight from the mean whole egg weight. The percentage of the egg weight comprised by the shell varies somewhat from species to species, but we used a value of 10% for all species. We then injected 1 µl of solvent/g mean egg contents. We did not make our injections based on the egg contents weight of individual eggs because of the much greater amount of time required to do it this way.

For air cell injections, we did not sterilize the solutions, but we did clean the glassware, stirring rods, vials, caps, pipettor tips, and other materials that might come in contact with the solvent. The inner shell membrane functions to exclude pathogens, and the high pH of the albumen and chemicals in the albumen protect against bacterial infections (Deeming 2002).

As the eggs were brought out of the incubator, we set them vertically in cardboard or plastic egg trays, wiped the cap with alcohol, drilled a hole in the cap of each egg, made the injections, and resealed the eggs with a hot glue gun. Others have used paraffin (Birge and Roberts 1976), tape (Walker 1967), or glue (Gilman *et al.* 1978) to seal the holes. All eggs in the same treatment were removed from the incubator together and were processed as a group. We kept the solvents in a refrigerator until we were ready to use them. To allow for the injected solvent to spread out over the inner shell membrane we warmed the solvents to 40°C, which is slightly higher than the temperature of the eggs in the incubator (approximately 37.5°C). By the time the solvent was drawn up into the pipettor and injected into the eggs, some cooling had taken place.

For air cell injections, we kept the eggs in a vertical position for a total of 30 minutes after being removed from the incubator. The time required for drilling and injecting seldom took longer than 5 minutes, with the remainder of the 30 minutes being allowed for the solvent to spread out evenly over the surface of the inner shell membrane. At the end of 30 minutes, we returned the group of eggs to the incubator, where, in all of our later experiments, the eggs rested on their sides and were rolled 180°/h.

Temperature and Humidity of the Incubator

Except for the gallinaceous species (chickens and pheasants), for which most incubator companies recommend a temperature of 99.75°F (37.6°C), we incubated the eggs of all other species at 99.5°F (37.5°C). During incubation, eggs lose weight through evaporative moisture loss. Most species are said to hatch best when this moisture loss, up to the point when the chick pips its way into the air cell, is approximately 15% of the original, fresh egg weight of the egg (Harvey 1993). Therefore, with each species, the relative humidity in the incubator has to be adjusted to achieve this degree of weight loss. Weighing the eggs, or usually a subset of the total number of eggs, once or twice a week and plotting the mean moisture loss can achieve this adjustment of relative humidity. In our early experiments, we used the uninjected controls to monitor egg weight loss and made adjustments in relative humidity that steered this group of eggs toward a 15% to 16% weight loss. We selected the uninjected controls because their survival is generally the best among all the various treatments and, therefore, the sample size that was available to plot the weight loss line was less likely to decrease with time. Later, we discovered that our reliance solely on egg weight information from the uninjected controls might have been inadequate to insure correct weight loss for the groups of eggs injected with corn oil. The injected corn oil coats some of the inner shell membrane of the egg and can impede moisture loss through the shell. To measure the degree to which corn oil impeded moisture loss, we injected groups of 17 to 19 mallard eggs with either no corn oil, 1 µl pure corn/g egg contents, or 2 µl pure corn oil/g egg contents. The eggs were injected after 4 days of incubation, and they were incubated on their sides and rolled 180°/h. During the course of 25 days of incubation, the uninjected control eggs lost a mean of 17.5% of their original weight, whereas the controls injected with 1 µl pure corn oil/g egg contents lost a mean of 16.4% of their original weight, and the controls injected with 2 µl of pure corn oil/g egg contents lost 14.7% of their original weight (Fig. 13). Our final protocol called for measuring moisture loss in the set of control eggs injected with 1 µl pure corn oil/g egg contents as well as in the set of uninjected controls. We checked and did not see evidence that the addition of methylmercury to the corn oil affected moisture loss any differently than did pure corn oil.

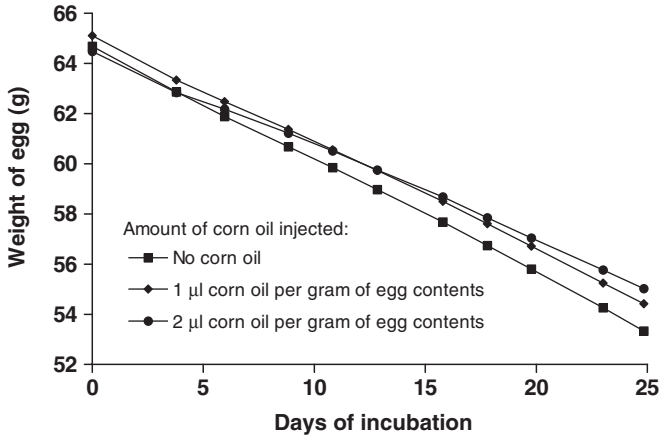

Fig. 13. Weight loss of mallard eggs when injected with no corn oil or with 1 or 2 µl corn oil/g egg contents

Passage of the Injected Methylmercury into the Egg

In addition to the studies we did with the passage of dyes into the egg, we conducted a study to quantify the amount of mercury that passed from an air cell injection into the albumen of eggs. Groups of 55 mallard eggs were randomized to controls or eggs injected with 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, or 3.2 µg/g mercury as methylmercury. The methylmercury was dissolved in corn oil, and each egg was injected into the air cell with 50 µl corn oil after 4 days of incubation. The eggs were incubated in the vertical position. Because measuring embryo survival of these eggs was part of another experiment, we collected for mercury analysis only eggs that had been culled out as dead. However, because we candled the eggs every 3 days, none of these dead eggs had a chance to decompose, and, consequently, we feel the use of dead eggs gave an accurate picture of mercury movement into the egg. After 7 days of incubation, which corresponded to 3 days after being injected, we collected 3 dead eggs from each group, except for controls and the 0.05- and 0.1-µg/g mercury groups, where none and only 1 and 2 eggs had died, respectively. To get control eggs, 2 eggs that died after 25 days of incubation were collected. To follow the progression of the mercury-treated corn oil into the egg, we saved 1 additional dead egg from 1 of the treatments (0.8 µg/g mercury) after 13, 19, 22, 25, and 28 days of incubation. In addition, from the 0.8-µg/g mercury group we saved 3 more eggs that died after 28 days of incubation, and analyzed the yolk and embryo (plus the remaining albumen around the embryo) separately. To accurately determine the fraction of the mercury dose that passed through the inner shell membrane and into the egg, we were careful not to tear the inner shell membrane when we emptied out the contents of the egg into sample

Table 2. Concentrations of total mercury in control eggs and eggs injected with 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, or 3.2 μg/g mercury as methylmercury chloride (mean with sample size in parentheses and extremes in brackets)

Mercury injected into egg (μg/g of egg contents, wet weight)[a]	Total mercury reported in egg (μg/g, wet weight)
0 (Control)	< 0.06 (2)
0.05	< 0.06 (1)
0.1	0.13 (2) [0.12 – 0.14]
0.2	0.19 (3) [0.12 – 0.27]
0.4	0.33 (3) [0.30 – 0.37]
0.8	0.58 (3) [0.48 – 0.74]
1.6	1.2 (3) [0.97 – 1.5]
3.2	2.5 (3) [2.3 – 2.8]

[a] Eggs from the groups injected with methylmercury were those that had been injected on day 4 of incubation and had died by day 7. No control eggs died by day 7, so 2 eggs that died on day 25 of incubation were analyzed. The egg contents excluded the inner shell membrane.

jars. Mercury concentrations in eggs were corrected for the moisture loss that occurs during incubation.

Concentrations of mercury in eggs are listed in Table 2. The two control eggs contained <0.06 μg/g mercury, which was the detection limit on a wet-weight basis. The single egg injected with 0.05 μg/g mercury that died by 7 days of age also was reported to contain <0.06 μg/g mercury on a wet-weight basis. By day 7, the percentage of the injected mercury that passed through the inner shell membrane into the egg for the other doses varied from a low of 73% for the 0.8-μg/g group to a high of 126% for the 0.1-μg/g group. The fact that more mercury was reported in the contents of the eggs injected with 0.1 μg/g mercury than was calculated to have been injected into this group of eggs is related either to the fact that some eggs within this group might have weighed less than the mean used to calculate the dose of injected mercury or to variation in chemical methods to detect mercury.

In the older eggs saved from the 0.8-μg/g mercury group, the concentrations of mercury that passed through the inner shell membrane into the contents of the egg after 13, 19, 22, 25, and 28 days of incubation were 0.81, 0.78, 0.87, 0.70, and 0.97 μg/g, respectively. Again, the values >0.8 μg/g may have been related to smaller-than-average eggs plus analytic variation. There was no consistent trend toward greater amounts of methylmercury passing through the inner shell membrane between days 13 and 28, but the average concentration found in the eggs during this period was 0.83 μg/g, which was higher than the value of 0.58 μg/g for the three eggs saved on day 7. We concluded that most of the injected mercury had passed through the inner shell membrane with all doses between day 4, when the injections took place, and day 7, when the first eggs were saved for mercury analysis, and that by day 13, essentially all of the mercury had passed into the egg.

In the set of three eggs injected with 0.8 μg/g mercury and opened after 28 days of incubation, the mean concentration of mercury in yolk was 0.47 μg/g, and the mean in the embryo plus remaining egg albumen was 0.80 μg/g. Tejning (1967) reported that in chickens fed methylmercury, approximately 95% of the mercury deposited in eggs was in the albumen, but by the time the egg was ready to hatch, approximately half of the mercury that was originally in the albumen had transferred to the yolk sac.

Design of the Final Injection Protocol

Based on the results of the studies we report in this article, we designed a final protocol that we have used to test the toxicity of injected methylmercury to the eggs of many species of wild birds. We will publish these results with wild bird eggs separately.

When a shipment of eggs comes in, we first wash the eggs in a disinfectant solution, dry them, and then weigh them as a group. From this group weight, we calculate the mean egg weight and multiply that value by 0.90 to arrive at an estimate of the mean egg contents weight. Any cracked eggs or eggs that have undergone incubation by the parents beyond the 3-day-old chicken stage are discarded. For each species, we use published information on the length of the incubation period to calculate the number of days of incubation that will equate to a 3-day-old chicken embryo. However, 1 day before the eggs are scheduled to reach the 3-day-old chicken stage, we candle the eggs to see if the embryonic development might have reached the desired stage faster than calculated. We continue to periodically candle the eggs until the embryos have the same appearance as a 3-day-old chicken embryo. Except in heavily pigmented eggs, where candling is difficult, we rely more on the appearance of the embryos than on the mathematic calculation of when a species is predicted to reach the equivalent of a 3-day-old chicken embryo.

Any eggs that are infertile or have died before reaching the appearance of a 3-day-old chicken embryo are discarded. The remaining eggs are randomized into the various treatment groups we have decided on. The number of groups depends mostly on the number of available eggs. We try to have a minimum of 10 eggs/treatment, with one group receiving pure corn oil without any added methylmercury. When we have enough eggs, we also have a group that does not receive the pure corn oil. After labeling the eggs, they are returned to the incubator to warm up. Once the eggs have rewarmed, we begin removing groups of eggs from the incubator for injection. We inject 1 μl corn oil/g egg contents. If we have such a group, we first remove the set of eggs that will be the uninjected controls. We set the eggs vertically, with the cap end up, in a cardboard or plastic egg tray and merely swab the cap of each egg with a 70% isopropyl alcohol swab. For the other groups of eggs we swab the cap with alcohol, drill a hole in the cap end of the shell with a rotary drill, and inject the eggs. The corn oil solutions are kept heated to 40°C until ready to use. We proceed through the treatments, starting with the corn oil controls and ending with the highest concentration of mercury. Using a repeating pipettor, we wipe the pipette tip with alcohol, draw the warmed corn oil up into the tip, inject each egg with the number of microliters of corn oil based on mean egg contents weight, and then seal the holes with a hot glue gun. Each group of eggs is allowed to remain in a vertical position at room temperature for 30 minutes, after which the eggs are returned to their sides in the incubator.

Eggs in the same treatment are grouped together in the incubator, but the location of that group of eggs is randomized as to its location in the incubator. Except for the eggs of gallinaceous species, which are incubated at 37.6°C, the eggs of all other species are incubated at 37.5°C. The eggs are placed on their sides and are rolled 180°/h, first in one direction and then in the opposite direction. The relative humidity is adjusted throughout incubation so as to produce an average weight loss in eggs up until the time of pipping of approximately 16%. We measure weight loss in the group of eggs injected with pure corn oil. If we have a set of eggs serving as uninjected controls, we monitor their weight loss separately because they tend to lose weight faster than corn oil–injected eggs. Two days before the eggs are scheduled to hatch, they are transferred to a separate hatching unit in which the temperature is decreased to 37.2°C, and the relative humidity is set to 70%. Eggs from each treatment are grouped together in separate hatching compartments.

At least twice a week we candle all eggs to determine embryo mortality. Dead eggs are removed from the incubator and opened to examine the embryo for deformities. However, we do not examine embryos that are <1 week old because it is very difficult to discern deformities in such a small embryo, especially when the early stages

of decomposition have taken place. After the eggs have hatched, we wait another 2 or 3 days and then open any unhatched eggs, examining them for deformities. Based on the appearance of the embryos, we can estimate how close they came to hatching. Because even artificially incubated control eggs of wild birds generally survive well up to the time they are placed in the hatching unit but often do not hatch as well as when incubated by the parents, the use of hatching success as the measure of the toxic effects of methylmercury weakens statistical tests. Therefore, we calculate the number of embryos that survived through 90% of the incubation period and use this as our measure of survival. Survival of controls through 90% of incubation can then be compared with that of the mercury-treated groups by a test such as Fisher's exact probability test, and dose-response curves and median lethal concentrations can also be calculated and compared among different species.

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