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Inheritance and Longevity of Infectious Pancreatic Necrosis Virus in the Zebra Fish, *Brachydanio rerio* (Hamilton-Buchanan)

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Zebra fish (*Brachydanio rerio*) were injected with infectious pancreatic necrosis virus (IPNV) and then spawned to determine whether the virus was passed on to the eggs, and if it was, how long it remained in the free-swimming F_1. The mating variations included parents receiving one or two injections of virus, and within these categories, matings in which both parents were treated or only one parent was treated. The results showed that transmission of IPNV to the egg did occur, and that this transmission was via the female alone. However, if the female was allowed to produce antibodies to the virus, as when she received two injections of IPNV, she transmitted the virus to the eggs for only a short period of time. In addition, when the virus was transmitted to the egg, it remained in the free-swimming F_1 for a period of at least 5 months.

The ovarian transmission of virus from adult to offspring has been of great concern for many years. Much of this work involves insects (4-8, 20). Perhaps the most widely studied example of this viral transmission is sigma particles in the fruit fly *Drosophila melanogaster* (14, 15). The CO_2 sensitivity of these flies, due to the presence of sigma, can frequently be transmitted by the female fly to her offspring, whereas the sensitive male fly, when mated to a nonsensitive female, produces sensitive offspring only occasionally (17).

Although the literature on transmission of virus in insects is quite vast (19), the literature on vertebrates is not as widespread. In 1936, Bittner (2) reported the discovery of a virus that caused mammary carcinomas in young female mice who had been suckled by mothers carrying a "milk factor." The offspring of mothers carrying this factor, when suckled by non-factor-carrying females, did not develop the tumors.

Transmission of virus to the F_1 by the semen is not unknown. Grieg (9) demonstrated that certain sheep viruses can be transmitted to the offspring via the sperm without infecting the female. It has been suggested that some of the offspring may acquire the virus from the father, who seems to carry a titer of this virus in the seminal vesicles (1). In addition, it has been shown that male carriers of this virus can transmit this virus to the female during copulation (3).

Wolf discovered that infectious pancreatic necrosis virus (IPNV), a virus that eventually causes mass mortality in trout, is often associated with the eggs of this species (22). The purpose of this present study is to determine whether this virus (IPNV) can be transferred from the parents to young in the zebra fish, a readily accessible and more suitable laboratory animal.

MATERIALS AND METHODS

Maintenance and assay of the virus. An extensive study of the virus began after the establishment of a permanent tissue culture cell line of rainbow trout gonadal tissue (RTG-2) (21, 23). This cell line has been used both to support the replication of IPNV and as a means of assaying this virus.

The RTG-2 cells were maintained and subcultured in Falcon plastic flasks in modified minimal essential medium-Earle base, after a modification by Rachlin et al. (16). A pancreatin-versene mixture (2.5% pancreatin and versene, 1:5,000 in a 1:24 ratio) was used to disrupt confluent monolayers for the purpose of subculturing. IPNV causes cytopathic effects in vitro similar to the inclusions observed in vivo (18). There are a variety of methods for determining viral titers. In our laboratory, it is standard procedure to follow the method of Karber (12).

Fish. The zebra fish, *Brachydanio rerio*, was maintained in the laboratory at 24 to 27°C. This fish species was initially chosen for these studies because of its ability to sustain a number of intraperitoneal injections of various antigens with minimum mortality, and because of their relative ease in handling and spawning in the laboratory (10, 11).

Transferal of virus from parent to egg. To determine whether IPNV could be transmitted from the

adults to the egg, and whether the ability to transmit this virus to the egg diminishes with time, fish were spawned for 6 weeks after the first or second injection of IPNV. Eight different spawning variations were involved:

(i) non-IPNV-injected male × non-IPNV-injected female (one injection)
(ii) IPNV-injected male × non-IPNV-injected female (one injection)
(iii) non-IPNV-injected male × IPNV-injected female (one injection)
(iv) IPNV-injected male × IPNV-injected female (one injection)
(v) non-IPNV-injected male × non-IPNV-injected female (two injections)
(vi) IPNV-injected male × non-IPNV-injected female (two injections)
(vii) non-IPNV-injected male × IPNV-injected female (two injections)
(viii) IPNV-injected male × IPNV-injected female (two injections)

In all experiments, IPNV-injected fish were injected with 0.5 ml of the virus in Hanks balanced salt solution (HBSS), at titers of $10^{7.1}$ viral particles per 0.1 ml. Fish receiving two injections were given the second injection 2 weeks after the first. Non-IPNV-injected fish were injected only with HBSS. All injections were made just posterior to the cardiac chamber. The angle of injection was toward the heart of the fish, which precluded any possibility of the gonads being accidentally injected.

Before spawning, the male and female zebra fish were sexed, separated into a number of different aquaria, and subsequently injected with IPNV. For the fish that were injected once, the spawnings commenced on day 2 after that injection and at 2-day intervals for a period of 6 weeks. Fish injected twice were spawned, beginning with day 2 after the second injection, and then spawned at 2-day intervals for a period of 6 weeks. No pair of fish was spawned more than once during the 6-week period. All experimental matings were run in triplicate.

Pairs of fish were spawned as recommended by LeGault (13). Each of the eight mating variations was spawned, and 100 fertile eggs from each mating were collected. The 100 eggs were placed in 1 ml of HBSS (the minimum amount of HBSS required for sonification) and then sonified at control output 4 (model W1400, Heat Systems Ultrasonics Inc., Plainfield, N.J.) for 1 h.

The mixture was then centrifuged at 300 × *g* for 1 h; then the liquid was decanted and passed through a bacterial filter (Millipore 0.22 μm, 13-mm diameter). This filtrate was titrated to determine viral concentrations per milliliter of filtrate. These figures were then used to calculate a relative determination of the virus in the eggs of the zebra fish.

Duration of the virus in the F_1. This series of experiments was designed to determine whether IPNV was further detectable in the free-swimming F_1. Based on the results of the previous experiments, four spawning variations were used in this series:

(i) non-IPNV-injected male × IPNV-injected female (one injection)
(ii) IPNV-injected male × IPNV-injected female (one injection)
(iii) non-IPNV-injected male × IPNV-injected female (two injections)
(iv) IPNV-injected male × IPNV-injected female (two injections)

HBSS-injected fish served as the non-IPNV-injected controls. Again, no pair of fish was spawned more than once. In the first two mating variations, the fish were spawned on days 2, 10, and 20 after the injection. In the last two variations, the fish were spawned only on day 2 after the second injection. All variations were spawned three times. The eggs were collected from each spawning and kept in aquaria, separate from the eggs of all other spawnings. The eggs were permitted to hatch, and one offspring was sacrificed each week for approximately 20 weeks. The experiment was done in triplicate, using the F_1 from the same spawning variation, but from different actual spawnings.

Because of the small size of these offspring, modifications in the previously described techniques were necessary. All young of all ages were sonified in 2 ml of HBSS. The sonified young were centrifuged, passed through a bacterial filter, and titrated as before. The amount of virus per milliliter of filtrate was then determined.

Statistics. All viral titers were expressed as the logarithmic exponent, i.e., $10^{5.1}$ is represented in all discussions as a titer of 5.1. Antibody titers were expressed as the reciprocal of the lowest concentration of test serum protecting RTG-2 cells from IPNV-caused cytopathic effects, i.e., protection at a serum dilution of 1/16 is represented as 16.

Where applicable, data have been averaged, and a standard error of the mean has been calculated. Each series of experiments was performed three times.

RESULTS

Transferal of virus from parent to egg. Virus in the eggs of male and female zebra fish that received one IPNV injection declined in titer from an average of 4.8, 2 days after the injection, to 3.36, 40 days after the injection (Table 1). When IPNV-injected females were mated with non-IPNV-injected males, the virus in the eggs had an average titer of 4.83 2 days after the injection, which declined to 3.17 after 40 days. In neither of these two spawning variations was virus evident from 42 to 44 days.

When the males were injected with IPNV and mated with noninjected females, detectable virus was never found in the eggs. Similar results were obtained when both parents were non-IPNV-injected.

Males and females that received two IPNV injections produced eggs that exhibited a viral titer that declined from an average of 4.63, 2 days after the second injection, to 2.96, 4 days after treatment (Table 2). There was no virus present in eggs produced after 5 days.

When the female was injected with IPNV twice and the male was noninjected, the eggs again showed viral titers that declined from 4.63, 2 days after the second injection, to 2.63, 4 days after this injection. No virus was found in the eggs produced, when examined 5 days after injection and up until the termination of the experiment at 14 days.

When only the males were injected with IPNV twice and the females were noninjected, or when both parents were noninjected, no detectable virus was ever found in the eggs.

Duration of the virus in the F_1. When the eggs of IPNV-injected parents, spawned on day 2 (day 2 F_1), day 10 F_1, and day 20 F_1 were allowed to hatch, virus was found in these F_1 up to 20 weeks after hatching in all cases (Table 3). The titers ranged from an average of 4.0 after the first week, to 2.9 after week 20 for the day 2 F_1, from 3.5 to 2.9 for the day 10 F_1, and 3.3 to 2.63 for the day 20 F_1.

The eggs of non-IPNV-injected males mated with IPNV-injected females were allowed to

TABLE 1. *Transfer of IPNV from parent to egg after one injection*

Day[a]	Viral titer[b]	
	Injected parents	Injected female
2	4.80 ± 0.07	4.83 ± 0.07
4	4.63 ± 0.07	4.76 ± 0.07
6	4.50 ± 0.20	4.63 ± 0.07
8	4.43 ± 0.18	4.56 ± 0.13
10	4.43 ± 0.07	4.50 ± 0.12
12	4.23 ± 0.07	4.30 ± 0.00
14	4.23 ± 0.07	4.30 ± 0.00
16	4.17 ± 0.07	4.17 ± 0.07
18	4.17 ± 0.07	4.17 ± 0.07
20	4.17 ± 0.07	4.23 ± 0.07
22	4.10 ± 0.12	4.23 ± 0.07
24	3.97 ± 0.13	4.37 ± 0.13
26	3.90 ± 0.12	4.23 ± 0.13
28	3.83 ± 0.24	4.30 ± 0.12
30	4.16 ± 0.07	4.17 ± 0.07
32	4.10 ± 0.00	4.23 ± 0.13
34	3.63 ± 0.07	4.10 ± 0.12
36	3.96 ± 0.07	3.57 ± 0.07
38	3.43 ± 0.18	3.23 ± 0.13
40	3.36 ± 0.13[c]	3.17 ± 0.07[c]
42-44	No detectable virus	No detectable virus

[a] Day after IPNV injection that fish were spawned.

[b] No detectable virus was found in any offspring of males injected once or of noninjected parents. Data are means of three samples ± 1 standard error of the mean.

[c] $P < 0.001$ between beginning of experiment and indicated data.

TABLE 2. *Transfer of IPNV from parent to egg after two injections*

Day[a]	Viral titer[b]	
	Injected parents	Injected female
2	4.63 ± 0.13	4.63 ± 0.07
3	4.10 ± 0.12	3.83 ± 0.07
4	2.96 ± 0.24[c]	2.63 ± 0.07[d]
5-14	No detectable virus	No detectable virus

[a] Day after IPNV injection that fish were spawned.

[b] No detectable virus was found in any offspring of males injected twice or of noninjected parents. Data are means of three samples ± 1 standard error of the mean.

[c] $P < 0.005$ between beginning of experiment and indicated data.

[d] $P < 0.001$ between beginning of experiment and indicated data.

TABLE 3. *Duration of IPNV in the F_1 of fish receiving one injection*

Week after spawning	Viral titer[a] at time of spawning after IPNV injection:		
	Day 2	Day 10	Day 20
1	4.00 ± 0.07	3.50 ± 0.12	3.30 ± 0.12
2	3.90 ± 0.12	3.50 ± 0.20	3.23 ± 0.18
3	4.03 ± 0.07	3.63 ± 0.13	3.30 ± 0.12
4	3.90 ± 0.12	3.50 ± 0.12	3.37 ± 0.13
5	4.03 ± 0.18	3.37 ± 0.27	3.10 ± 0.12
6	4.03 ± 0.07	3.30 ± 0.12	3.23 ± 0.07
7	3.97 ± 0.18	3.50 ± 0.20	3.10 ± 0.12
8	3.73 ± 0.07	3.50 ± 0.12	3.30 ± 0.12
9	3.70 ± 0.12	3.37 ± 0.13	3.37 ± 0.13
10	3.63 ± 0.18	3.30 ± 0.00	3.30 ± 0.00
11	3.63 ± 0.07	3.43 ± 0.18	2.97 ± 0.07
12	3.10 ± 0.00	3.50 ± 0.20	2.90 ± 0.12
13	3.03 ± 0.07	3.57 ± 0.07	2.70 ± 0.12
14	3.10 ± 0.12	3.50 ± 0.12	2.70 ± 0.20
15	3.23 ± 0.07	3.43 ± 0.18	2.83 ± 0.27
16	3.10 ± 0.12	3.10 ± 0.00	2.90 ± 0.12
17	3.03 ± 0.07	3.10 ± 0.20	2.70 ± 0.12
18	2.90 ± 0.12	2.97 ± 0.07	2.33 ± 0.15
19	2.70 ± 0.20	2.97 ± 0.18	2.50 ± 0.20
20	2.90 ± 0.12[b]	3.10 ± 0.12	2.63 ± 0.07[c]
21		2.90 ± 0.12[d]	

[a] Mean of three samples ±1 standard error of the mean.

[b] $P < 0.005$ between beginning of experiment and indicated data.

[c] $P < 0.01$ between beginning of experiment and indicated data.

[d] $P < 0.025$ between beginning of experiment and indicated data.

hatch, and virus was found in all of these F_1 for a period of 19 weeks (Table 4). In addition, virus was found in the eggs of day 2 and day 20 spawnings for 21 weeks, after which the experiment was terminated. The day 2 titers ranged from an average of 3.63 to 2.5, and the day 20 titers ranged from an average of 3.3 to 2.7.

When the eggs of parents that were injected twice with IPNV and spawned on day 2 after the second injection were allowed to hatch, detectable virus was found in these F_1 for a period of 17 weeks. The titers ranged from an average of 3.57, after 1 week, to 3.03, after 17 weeks (Table 5).

The eggs of non-IPNV-injected males and females injected twice with IPNV were spawned 2 days after the second injection. When allowed to hatch, there was virus in the F_1 for a period of 19 weeks, ranging in titer from an average of 3.76, after 1 week, to 3.1 at the conclusion of the experiment at 19 weeks.

DISCUSSION

Transferal of virus from parent to egg. With the exception of fish spawned 34 and 36 days after a single injection of IPNV, the virus found in the eggs of non-IPNV-injected males mated with females injected once with IPNV as well as in the eggs of parents injected once with IPNV was remarkably constant between the two types of offspring (Table 1). There was no significant difference in the virus titer found in each of the spawning variations, when compared with each other, except for the 34- and 36-day spawns of both variations, which were significantly different. The reason for this 34- and 36-day discrepancy is not clear at this time. No virus was found in the eggs produced 42 and 44 days after the first injection by either spawning variation.

The eggs of males injected once with IPNV mated with non-IPNV-injected females and the eggs of non-IPNV-injected parents never showed detectable virus. This is significant in light of the above data on IPNV-injected females. It would seem that this virus is transmitted to the eggs only when the female parent is injected. If the male alone is injected, and the female not injected, there is no transmission of virus to the egg. Further, since the titers of virus found in the eggs of IPNV-injected parents are not significantly different from the titers of virus in the eggs of IPNV-injected females and non-IPNV-injected males, it can be concluded that the female alone is responsible for viral transmission to the F_1.

It was interesting to note that no virus was transmitted to the eggs produced 42 to 44 days postinjection by either the IPNV-injected fe-

TABLE 4. *Duration of IPNV in the F_1 of female fish receiving one injection and noninjected males*

Week after spawning	Viral titer[a] at time of spawning after IPNV injection:		
	Day 2	Day 10	Day 20
1	4.10 ± 0.12	3.63 ± 0.27	3.30 ± 0.20
2	4.37 ± 0.07	3.70 ± 0.23	3.50 ± 0.20
3	4.03 ± 0.07	3.43 ± 0.18	3.50 ± 0.23
4	4.10 ± 0.12	3.50 ± 0.23	3.30 ± 0.20
5	3.77 ± 0.07	3.50 ± 0.50	3.43 ± 0.18
6	3.90 ± 0.12	3.36 ± 0.18	3.50 ± 0.23
7	3.90 ± 0.23	3.36 ± 0.07	3.30 ± 0.20
8	3.70 ± 0.31	3.30 ± 0.31	3.10 ± 0.20
9	3.83 ± 0.32	3.50 ± 0.31	3.36 ± 0.27
10	3.63 ± 0.18	3.57 ± 0.29	3.10 ± 0.35
11	3.63 ± 0.18	3.50 ± 0.12	3.17 ± 0.07
12	3.70 ± 0.31	3.37 ± 0.18	3.42 ± 0.18
13	3.83 ± 0.18	3.30 ± 0.20	3.30 ± 0.00
14	3.70 ± 0.23	3.43 ± 0.18	3.03 ± 0.13
15	3.70 ± 0.12	3.10 ± 0.31	2.97 ± 0.13
16	3.30 ± 0.12	2.97 ± 0.18	3.03 ± 0.13
17	3.43 ± 0.18	2.90 ± 0.40	2.77 ± 0.18
18	3.10 ± 0.12	2.50 ± 0.31	2.83 ± 0.07
19	3.03 ± 0.13	2.50 ± 0.35	2.77 ± 0.18
20	2.97 ± 0.18		2.83 ± 0.07
21	2.70 ± 0.12[b]		2.70 ± 0.12

[a] Mean of three samples ±1 standard error of the mean.

[b] $P < 0.005$ between beginning of experiment and indicated data.

TABLE 5. *Duration of IPNV in the F_1 of fish receiving two injections*

Week after spawning	Viral titer[a]	
	Both parents injected	Female only injected
1	3.57 ± 0.24	3.76 ± 0.18
2	3.57 ± 0.24	3.76 ± 0.29
3	3.50 ± 0.12	3.50 ± 0.12
4	3.42 ± 0.18	3.90 ± 0.31
5	3.30 ± 0.12	4.03 ± 0.07
6	3.36 ± 0.13	4.03 ± 0.18
7	3.56 ± 0.29	3.90 ± 0.00
8	3.50 ± 0.12	3.70 ± 0.31
9	3.50 ± 0.23	3.57 ± 0.13
10	3.57 ± 0.24	3.77 ± 0.13
11	3.30 ± 0.12	3.70 ± 0.13
12	3.57 ± 0.29	3.63 ± 0.13
13	3.23 ± 0.24	3.56 ± 0.29
14	3.10 ± 0.12	3.37 ± 0.13
15	2.90 ± 0.12	3.50 ± 0.20
16	3.03 ± 0.07	3.30 ± 0.20
17	3.03 ± 0.18	3.23 ± 0.24
18		2.97 ± 0.18
19		3.10 ± 0.12[b]

[a] Mean of three samples ±1 standard error of the mean.

[b] $P < 0.05$ between beginning of experiment and indicated data.

male or parents (Table 4), although it has been demonstrated that virus is present in the female gonad during this period at titers over 3.0 (unpublished observation).

The female gonadal titers decreased by 1 log after the first injection of IPNV from the period beginning 5 h after the injection to 33 days postinjection. The gonadal virus titer decreased an additional 1 log from day 33 to 42 postinjection. This would indicate a buildup of antibody at an accelerated rate in the later postinjection period. Presumably, sufficient antibody was present in the gonads at 42 to 44 days to neutralize the virus that would normally be found in the egg, but for some reason that remains unclear at this time. The gonadal virus itself is not yet completely neutralized and was not neutralized until 64 days after the injection of IPNV.

When parents injected twice with IPNV and spawned, or when non-IPNV-injected females were mated with males injected twice with IPNV, detectable virus was never found in the eggs. This was expected, since it has been shown that only the female injected once with IPNV transmits detectable IPNV to the eggs. This confirms the previous finding that the injected males do not play a role in transmitting virus to the egg.

When parents injected twice with IPNV, and females injected twice with IPNV mated to non-IPNV-injected males were spawned, virus was transmitted to the eggs (Table 4). However, the period of virus transmission ceased after 4 days, and no virus was detected, ever, after 4 days after the second injection. This closely parallels the duration of virus in the gonads of female fish injected twice with IPNV (unpublished observation). There, too, the gonadal virus was present for only 4 days after the second injection. This, plus the statistical insignificance of the viral titers in the eggs of these twice-IPNV-injected variations, and the fact that there is no detectable virus in the male gonads after two days in twice-IPNV-injected males, further establishes that only the IPNV-injected female transmits detectable virus to the eggs.

Duration of the virus in the F_1. The duration of the virus in the F_1, both parents of which received a single injection of IPNV, follows a pattern of decreasing titers in all cases (Table 3). The F_1 generation, the product of fish spawned 2 days after injection, demonstrate a significant viral decrease from week 1 to week 20. However, the week-to-week changes in titer are not significant in any instance. When the experiment was terminated after week 20, there was still considerable virus in the day 2 F_1 generation. The F_1 generation, the product of day 10 postinjection spawning of the adults, demonstrated a significant decline in viral titers from week 1 to week 21. The day 20 F_1 generation also showed a significant decrease in viral titers from week 1 to week 20, when the experiment was terminated (Table 3). The most important point here is not that the virus decreases weekly in the F_1, but rather, that the virus is passed from the IPNV-injected adults through the eggs and, when the eggs hatch, are found in the free-swimming F_1 for a period of 4 to 5 months. This transfer of virus to the free-swimming F_1 has never been demonstrated in fish before.

When only the adult females are given a single injection of IPNV and mated with non-IPNV-injected males, the F_1 generation also shows appreciable virus titers that decrease with time (Table 4). The decrease is significant only for the day 2 F_1 generation. Again, it must be emphasized that the major point is the presence of the virus for so many weeks in the F_1.

When both parents are injected twice with IPNV at two-week intervals and spawned on day 2 after the second injection, virus is again found in the free-swimming F_1 (Table 5). The same is true if only the female is injected twice with IPNV. Virus titers decline with respect to time in both cases, but are significant only in the case of the progeny of IPNV-injected females mated to non-IPNV-injected males. The F_1 weekly titers, when contrasted for the above two spawning variations, are in close agreement.

The viral titers of the day 2 F_1 of twice-IPNV-injected parents and female only IPNV-injected (Table 5) are in close agreement with the titers of F_1 generation whose parents received only a single injection of IPNV (Table 3). The day 2 F_1 of Table 5 are also in close agreement with the titers of the F_1 generation of Table 4, in which the female is IPNV-injected and the male is not.

These data are evidence for the transferal of IPNV from the female zebra fish to young via the egg. The male appears to play no role in this transmission. In addition, the virus is maintained in the young for a period of at least 5 months.

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