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SHORT COMMUNICATION

# Egg microinjections – method experimental viral infections in brook trout *Salvelinus fontinalis* (Mitchill, 1815) and rainbow brook trout *Salvelinus fontinalis* ♂ × *Oncorhynchus mykiss* ♀

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**Abstract.** Microinjection is a micromanipulation technique that has been used in various fields and in a variety of species, including teleost fish, for over a hundred years. Microinjection is mainly used in genetic, molecular, and toxicological research. The paper describes an experimental study of a microinjection technique for the administration of Infectious Pancreatic Necrosis Virus (IPNV) in fertilized eggs of brook trout, *Salvelinus fontinalis* (Mitchill), and a rainbow trout *Oncorhynchus mykiss* (Walbaum) hybrid, *Salvelinus fontinalis* ♂ × *Oncorhynchus mykiss* ♀. The oocytes and embryos of these salmonids have complex structures that require modifying the method commonly used in zebrafish, *Danio rerio* (Hamilton), research. A modular apparatus and technique for injecting brook trout and rainbow brook trout fish embryos with IPNV are described. The modified microinjection method can be used successfully to administer to salmonid eggs infectious substances that simulate the vertical transmission of pathogens.

**Keywords:** Infectious Pancreatic Necrosis Virus, IPNV, microinjections in fish, trout hybrids, salmonid hybrids

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## Introduction

Microinjection is a micromanipulation technique invented in 1902 by Dr. Marshall Barber that is currently instrumental in biomedical research (from transgenics, gene targeting, animal cloning, human infertility treatment, and nuclease-guided genetic engineering to RNA-guided genome editing) and healthcare. In aquaculture, microinjections are used in genetic, molecular, and toxicological research (Metcalfe and Sonstegard 1984, Black et al. 1985, Metcalfe et al. 1988, Inoue 1992, Bailey et al. 1996, Okhyun et al. 2015, Goto et al. 2019, Michiels et al. 2019). Microinjection is a very precise technique, which permits inserting/retrieving substances at a microscopic or macroscopic level into/out of a single living organelle, cell, or multicellular entity using a needle, usually a glass micropipette 0.5 to 15 μm in

diameter. One of the main advantages of microinjection is the wide variety of substances that can be delivered, such as DNA, RNA, protein, and other macromolecules (Elaswad et al. 2018, Goto et al. 2019, Xu 2019). Salmonid microinjections described in the literature have many applications: genetic manipulation (mainly production of transgenic fish), in vitro fertilization, cryopreservation technology, and toxicological tests (Bailey et al. 1996, Shirakashi et al. 2015). Moreover, thanks to the possibility of administering precise doses, the microinjection method can also be used to administer other substances, including carcinogens (Black et al. 1985) or infectious agents, into eggs and embryos, to simulate the vertical transmission of pathogens.

Infectious pancreatic necrosis (IPN) is a disease of many freshwater and saltwater fish species and is caused by the ubiquitous RNA virus belonging to the family Birnaviridae, genus Aquabirnavirus (Reno 1999, Dopazo 2020). The disease is not listed by the World Organisation for Animal Health even though it contributes to significant losses in farmed and wild salmonids worldwide (Silim et al. 1982, Taksdal et al. 1997, Bebak et al. 1998, Barrera-Mejía et al. 2011). Infectious pancreatic necrosis virus (IPNV) is transmitted not only horizontally, but also vertically through sex products of fishes with latent infection (Bootland et al. 1991, Reno 1999). The most susceptible fish stage to vertical transmission is freshly hatched fry (Mulcahy and Pascho 1984, Bootland et al. 1991, Reno 1999, Mutoloki et al. 2016). In this study, IPNV was chosen as the infectious agent to simulate vertical infection by the microinjection of the virus in fertilized eggs.

Eggs of teleost fishes, including salmonids, have a complex multi-layered structure with two distinct barriers with very different physical properties. They are surrounded by a hard extracellular coat (referred to as a chorion, outer eggshell, zona pellucida), composed of cross-linked polypeptides that provide mechanical protection to the embryo during development. The second barrier is the vitelline membrane (lipid bilayer) that surrounds the yolk. Since these egg coats play important roles during oogenesis, fertilization, and early embryogenesis (Darie et al. 2005, Berois et al. 2011, Shirakashi et al. 2015), salmonid eggs preferably are not dechorionized before microinjection. Therefore, the needle used for the procedure has several barriers to penetrate and requires the application of adequate capillary pressure to overcome ooplasmic pressure. The main factors that affect the outcome of microinjection are the nature of the yolk cell, the buoyancy of the eggs, the width of the perivitelline space, the hardness of the chorion, the diffculty of removing the egg chorion, and the stickiness of the chorion (Goto et al. 2019). The initial quality of the oocytes is also important; this can be estimated easily by observing the distribution of lipid droplets beneath the oolemma on the surface of the protein yolk (Craik and Harvey 1984, Mansour et al. 2007). Moreover, fixing and positioning eggs is required so the blastodisc faces the microinjection needle. Furthermore, the eggs might have high internal pressure, and failed injection attempts may break the tip of the glass needle and cause the egg material to retract, which can interfere with normal embryonic development (Goto et al. 2019).

The study aimed to establish a technical protocol and evaluate the use of the microinjection technique for experimental viral infection in farmed fish.

## Materials and Methods

### Eggs and virus

A total of 1,728 oocytes striped from female brook trout and rainbow trout were fertilized with brook trout sperm with the standard procedure to obtain all-female triploids of brook trout and rainbow brook trout interspecific hybrid at Dabie Hatchery, Poland. The eggs were then transported under temperature-controlled conditions to the laboratory and divided into three main research groups: a control group consisting of untreated eggs, a placebo (virus-free vehicle) injected group, and an IPNV-injected group. The Sp (Spjarup) reference strain of IPNV obtained from the National Veterinary Research Institute (NVRI) in Poland (GenBank accession number: AM889221) was prepared. The

infectious virus titer in the solution was determined using a fifty-percent tissue culture infective dose ($TCID_{50}$) and was $1 \times 10^8$ $TCID_{50}$ $ml^{-1}$. The viral medium (RPMI, Sigma-Aldrich) contained a phenol red dye that facilitated the optical control of microinjection.

## Microinjection equipment

The eggs were injected under a binocular stereoscopic microscope (Delta Optical). Two sets of equipment were tested. The first one, used in the pilot study, was the InjectMan NI2 system with FemtoJet Express (Eppendorf) with glass pipettes. Glass pipettes for microinjection were made from single-channel borosilicate glass capillaries with reinforcement filament (outer diameter 1.00 mm, inner diameter 0.78 mm, length 10 cm). The capillaries were heated and pulled with a puller for micropipettes (Puller P-1000, Sutter Instrument), sharpened (Narishige EG-400 microgrinder), and forged (Narishige MF-900 microforge). The second system, used in the main study, contained a 10 µl single-channel borosilicate glass syringe (outer diameter 6.60 mm, inner diameter 0.46 mm) mounted with a repeating semi-automatic dispenser for syringes (Hamilton) with stainless steel needles with an SN-RN connection, a size of 26sG (outer diameter 0.47 mm, inner diameter 0.13 mm, length 19.0 mm), angle of 30°, and point style 4 (Hamilton). During injection, eggs were held by placing them in a 96 well plate with round bottoms partially filled with agar gel (Fig. 1). Each well was approximately the same diameter and slightly shallower than the egg diameter. The plates were placed one at a time in a glass tank mounted on a thermoelectric Peltier cooler plate module (Fig. 2), which allowed controlling the water temperature during the procedure.

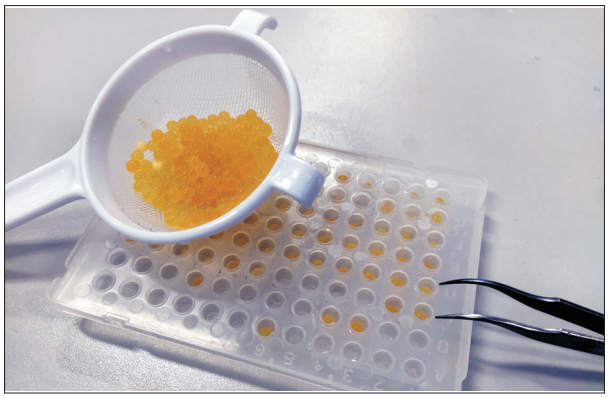

Figure 1. Test of the agar gel filled plate – the amount depicted in the photograph is insufficient to properly support the eggs.

## Impact of microinjection on embryo development

In the main experiment, 2,304 eggs were injected in less than 7.5 h after fertilisation with 0.2 µl of placebo solution or viral solution (no more than 0.6% of the volume of a single egg). Following microinjection, each of the main research groups was placed in separate horizontal hatching troughs made of smooth glass fiber reinforced polyester with four boxes each that was divided into four subgroups of two boxes

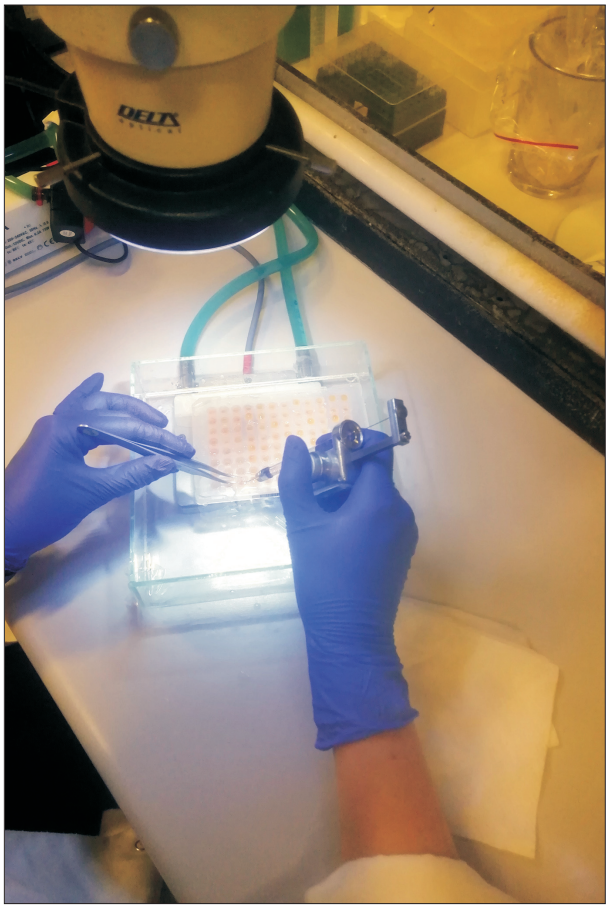

Figure 2. Agar gel and a plate filled with eggs in a glass tank mounted on a thermoelectric Peltier cooler plate module.

per species. There were 12 experimental subgroups of 288 eggs each for a total of n = 3,456 eggs. Then embryos were incubated in a water temperature range of 9-11°C. Dead embryos were counted and picked once daily at a fixed time.

## Results and disscusion

To develop an efficient protocol for the microinjection of brook trout and rainbow brook trout hybrid eggs, we conducted a series of experiments aimed at optimizing the method of mounting eggs and selecting the appropriate pressure parameters.

### Automatic microinjections with a glass needle

The puncture force with glass capillaries needed to make a critical puncture force for chorion breakage was higher than the durability of the glass needle. This meant the needles broke very quickly, but they also caused significant deformities in the chorion and leakage of yolk plasma through the puncture hole into the perivitelline space. Shirakashi et al. (2015) demonstrated that egg deformation caused by an injection needle resulted in a significant nonlinear increase in internal pressure within the egg, which might be relevant to the loss of the integrity of the embryonic cell membranes. Therefore, the correct pressure parameters required for microinjecting with an InjectMan NI2 system with FemtoJet Express (Eppendorf) could not be identified and no replicable dosage of virus solution could be achieved.

### Semi-automatic microinjections with a stainless steel needle

A stainless steel needle size 26sG has half the inner diameter of the standard 26-gauge needle and nearly double the wall thickness (0.18 mm compared to 0.10 mm). These parameters combined with a 30° bevelled needle point were enough to pierce through egg membranes without causing deformities in the chorion or leakage of yolk plasma in most eggs. Hydrostatic pressure provided by the syringe did not permit suction of the solution from the syringe, and the semi-automatic dispenser made it possible to microinject repeatable doses in each egg. One needle was sharp enough to microinject one fully loaded 96 well plate of fertilized eggs. The presence of agar gel in the wells prevented accidental needle blunting from walls while ensuring proper stabilization.

### Mortality of brook trout and hybrid embryos after microinjections with a stainless steel needle

Four hours after microinjections, 100% mortality was observed in the glass needle procedure, compared with no mortality in all groups of the stainless steel needle procedure. The influence on viability and mortality in groups without microinjections compared to ones with placebo and IPNV are shown in Fig. 3. For 14 days after fertilization, there were no distinct differences observed that could have been caused by microinjection.

Microinjection methods used commonly in zebrafish are not applicable for teleost oocytes and embryos surrounded by a hard chorion, which is why there is a growing need for developing new methods for more species (Shirakashi et al. 2015, Goto et al. 2019). A technique for the microinjection of salmonid fish embryos with a viral solution is described, with the key feature of the relative simplicity of routine through-the-eggshell injection into the early blastocyst-stage egg. The procedure is rapid and sufficient for two persons working as a team (one mounting eggs on a plate, while the second one does the microinjection), which means 96 injections of embryos per half hour. Microinjection with a stainless steel needle induced low mortality per se, i.e., optimal net survival rates were comparable to controls not given any injection, regardless of whether they contained the virus or not. Because only small volumes of the virus are handled in an exact known

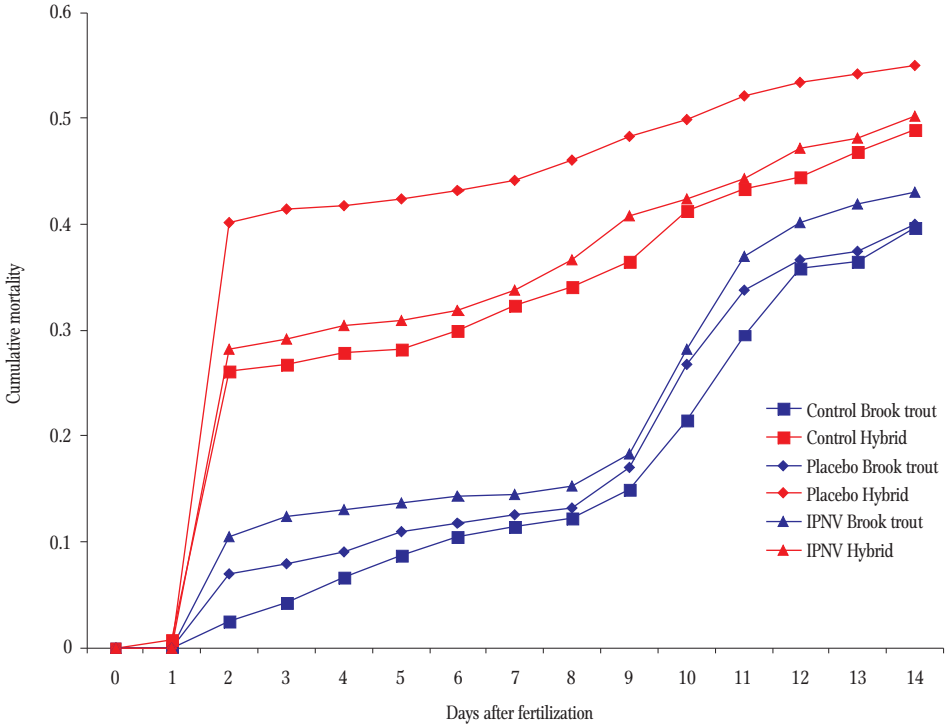


Figure 3. Chart of cumulative mortality over 14 days after microinjections.

amount, the exposure method reduces the risks of contamination to both the equipment and the environment. Despite the microinjection method being more intensive with regard to time, effort, and equipment than experimental infection through immersion, it can be used to rapidly administer a broad range of solutions that cannot penetrate the chorion in immersion, including dyes, drugs, cryoprotectants, and infectious agents.

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