

Icubation of Eggs

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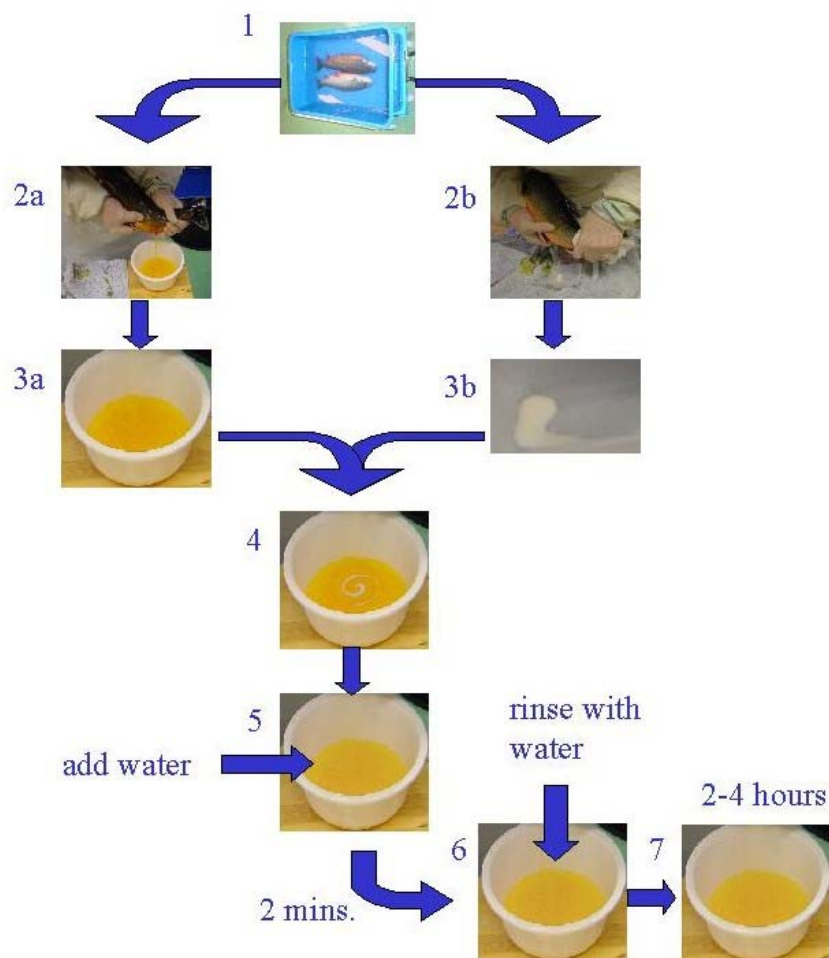
Content

This protocol starts with the stripping of the eggs. You will also find information about embryonal development and a selection of different types of incubators, together with the actual protocol on incubation of eggs - procedures and environment. Procedures will in this context mean handling and work involved whereas environment describes holding conditions as i.e. temperature. The protocol on care of broodstock will give information of earlier stages of reproduction.

Stripping of fish and fertilization of eggs.

An overview of the consecutive steps in the stripping process is shown in the figure below.

The process step by step.



1. Anaesthetize the fish. Arctic charr is very hardy towards different types of anaesthetics. Use what you are accustomed to, and follow the dosage advised by the manufacturer. Remember that the brood stock is the most valuable fish you have. We have used Benzocaine (50 ppm for 3-4 mins.) for several years on charr, and that works fine. Many prefer MS-222 (tricaine), but remember that it reduces pH, so you need to buffer in fresh water.

2a. Dry off surface water using a soft cloth. Removal of skin mucus may lead to fungal infections later. Check that the eggs are released freely before you start collecting the eggs. It is better to waste a few eggs than to get contaminants in the egg batch. Gently stroke the buccal cavity from behind the pectoral fins backwards to the vent (anus). This needs to be repeated several times. Do not use much force, as this will damage the internal organs.

3a. Each female will give approximately 2000 eggs per kilo fish. The quality of the eggs usually improve from first to second time spawners. The eggs should be stored dark and cool (4-6 degrees celsius) while collecting the milt.

2b. As with females, dry off surface water using a soft cloth. Males are more susceptible of contracting fungal infections than females. The males are stripped as the females, but you only have to work with the caudal area of the buccal cavity. Waste the first few drops to get rid of urine and contaminants. It is usual to combine milt from three or more males for each batch of eggs. The milt to egg ratio should at least be 3 ml milt per litre of eggs to ensure sperm cells in excess.

3b. Each male will give only a few millilitres of milt. There is usually not much time to check the quality, as the sperm cells only stays active for 60-120 seconds. Check for contaminants. The milt should have a creamy appearance.

4. Mix the milt gently with the eggs. Make shure the milt is evenly distributed in the egg batch. The ovarian fluids will at this point reduce the concentration of the inhibiting potassium in the milt so that the sperm cells becomes active and fertilisation begins.

5. After 30-60 seconds (dependent on temperature) add some water to the eggbatch, mix it genly and leave for another 2 minutes. Use water with the same temperature as the holding water of the broodstock. The water will dilute potassium even more, and sperm cell activity gets a second burst. This improves the fertilisation ratio.

6. Rinse the eggs with clean water to remove excess milt.

7. Leave the eggs to harden their shell for 2-4 hours. They can also be moved directly to the incubators to harden there. The entering of a spermcell into the egg activates a series of actions (cortical reaction), involving uptake of water and increased size (approx. 30%), that eventually ends with hardening of the egg.

After the eggs have hardened they can be moved and handled for approximately 24 hours (although some i.e. Jonston, 2002 recommend transfer to incubators immediately after fertilization). From this point and until the eyed stage, the eggs should be handled as little as possible.

At this stage incubation begins.

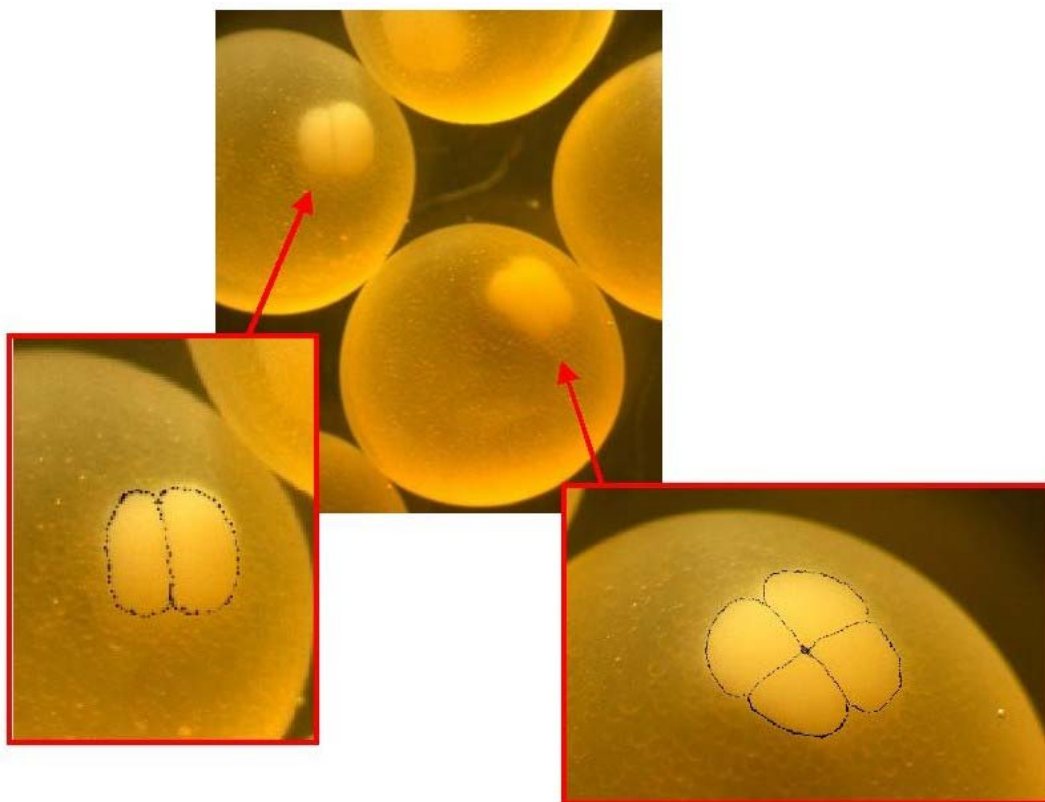
Embryonal development -

From fertilisation of eggs to free swimming alevins.

Development of eggs can be divided into three stages: Cleavage, Epiboly and Organogenesis.

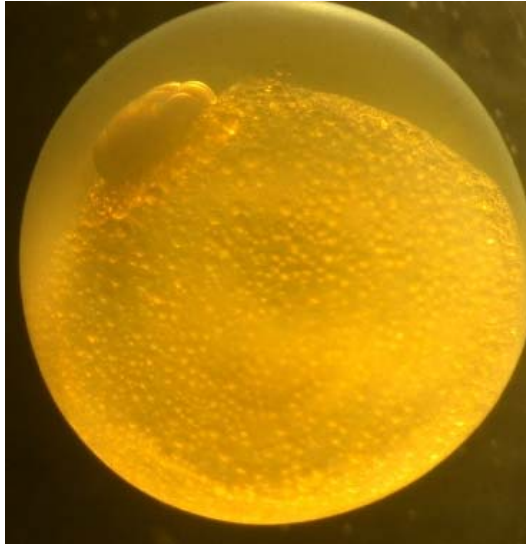
Before development starts, the eggs have to be fertilized, which is the process of one spermatozoan entering the egg through the micropyle. This initiates an activation of cortical alveoli - known as cortical reaction, or water hardening. The vitelline membrane enveloping the egg is replaced by a new membrane that is soon to harden and form the new chorion. The egg takes up water and increases in size by approximately 30%. The new chorion protects the embryo from physical injury and pathogens.

All eggs, fertilized or not, will react when in contact with water and start water uptake and swelling. Eggs should therefore not be exposed to water before fertilization has taken place.



Arctic charr eggs in early cleavage stages. The pictures in red frames show 2 cell stage (left) and 4 cell stage (right) with the cells outlined. (Photo: Marianne Frantzen, NFH, University of Tromsø).

Cleavage. The embryo starts developing after fertilization. First the cytoplasm, a colorless cell fluid, moves over the surface of the yolk. It concentrates at the animal pole where it rounds up and rises slightly to form a hemispherical dome. This is the first cell of the embryo, known as the blastodisc. Cell division starts with the first cleavage of the blastodisc to form two cells (the two cell stage - see picture above). Each of the two new cells will divide to form four cells (the four cell stage), and this will continue with these four dividing into 8 cells and further to 16 - 32 - 64 - 128 and so on. It is envisaged to check fertilization rate early at this stage (at four to eight cell stage). Later, the cells will be too small for detection under low magnification. As cleavage goes on the cells become smaller and smaller. After the 32 cell stage the morula stage is formed (picture below). The individual cells may still be seen in the granular appearance of the morula, but they are difficult to count.



Arctic charr egg in the 32 cell stage. The cluster of cells is on the upper left side in the egg. The small round shapes in the yolk are oil droplets. (Photo: Marianne Frantzen, NFH, University of Tromsø).

Epiboly. The formation of the early embryo initiates the second stage. During this stage the cells formed in the cleavage phase starts to specialise to form tissues. The edge of the blastodisc expands and grows down, covering the surface of the yolk. The overgrowing edge, sometimes called the germ ring, eventually envelopes the yolk - this process is the actual epiboly (=growth of one part over another). The tissue formed by this germ ring will become the yolk sac and later the tissues enclosing the body cavity. During this process, the organisation of the cells become more clear. The head of the developing embryo is at the original animal pole, and the beginning of the tail is near the border of the germ ring.

The head can be seen when 1/3 of the yolk is overgrown by the germinal layer. Future muscle tissues are formed in the body region on either side of the spinal cord. Optic vesicles - the developing eyes - are visible at 1/2 epiboly and the eyes are formed at 2/3 epiboly. Dependent on temperature, epiboly is completed within a few days when the edges meet at the opposite side of where it started - enclosing of blastopore. A new membrane of cellular epithelium now envelops the yolk. Handling of eggs during epiboly must be avoided. Even the slightest movement of eggs may cause the membrane to leak, leading to loss of salts and denaturation of cytoplasm proteins. The end result is reduced survival of eggs. At the end of this stage the cells formed during cleavage have turned into tissues which forms the basic structures of the embryo.



The picture above show eggs in the eyed stage. Note the small dark dots within the eggs, these are the pigmented and developed eyes. The white eggs are dead and should be removed to avoid fungal infections.

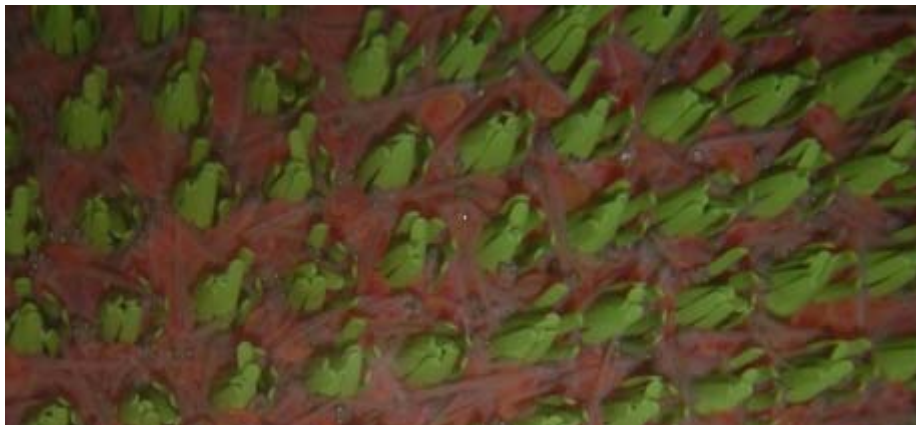
Organogenesis. Internal organs occur during organogenesis. When the epiboly is complete the posterior end of the embryo extends and lifts free from the surface of the yolk. This is the beginning of formation of the caudal fin. Brain, eyes, neural arch, muscles (myomeres), heart, circulatory system, intestines and liver are developing. Blood vessels are covering the surface of the yolk, a development called vascularisation. When 3/4 of the yolk is vascularised the head is free, and the eyes are fully pigmented. This stage is easily recognised by the two dark spots within the eggs - the eyes - hence eyed stage. The heart starts to beat, and later during this stage the muscle activity increases. The embryo is fully pigmented and complete, but do not hatch.

The eggs are now hardy and handling of individual eggs is possible. It is important to remove dead eggs. Fungal infections spread easily and may cause problems (and death) to live eggs. Oxygen consumption increases.



Newly hatched alevins and eyed eggs waiting to hatch. Although the eggs have been stripped at the same time and kept under identical conditions, hatching may still last over a few days.

At hatching the muscular activity of the fully developed embryo, now referred to as alevin, increase and eventually cause the eggshell to break open. The alevin will immediately move downwards and seek for shelter. Further development is for some weeks onwards only supported by nourishment and energy from the yolsac. To ensure that as much of the energy as possible is allocated to growth and development, alevins should be provided with some sort of shelter and support (e.g. biomaths). This minimises the amount of energy spent on movement and holding an "upright" position. The alevins stay in this shelter until 70-80 % of the yolk is used before they start moving towards the surface - "swim-up" stage. When reaching the surface they snap a small amount of air for their swimbladder to aid buoyancy, and they are now ready to search for and ingest external food. The swim-up is a certain sign that the alevins are ready to start feeding. As with hatching, swim-up is not very synchronised so it is envisaged to try feeding the alevins a few days before this behaviour starts.



Alevins embedded in a commonly used substrate - "astroturf". This provides some shelter and support to the alevins, helping them to hold an upright position with minimal energy expenditure. The alevins are using the stored energy to grow and develop maximally before "swim-up" and startfeeding.

INCUBATION OF EGGS - procedures and environment

This protocol starts at the point when the fertilised eggs are transferred to the incubators. To read more about fertilization, check the link on the content page of this protocol.

After the eggs are fertilized they undergo a water hardening process (See [embryonal development](#)). Mechanical disturbances during this period may cause severe damage to the egg and result in reduced survival rate. The eggs should therefore be transferred to the incubators either before or after the water hardening process. If they are transferred before water hardening this should take place immediately after last rinsing with water when the excess sperm was removed. If you decide to wait, the eggs should be set aside for 3-4 hours without disturbance. After the water hardening is complete the eggs are quite hardy for a period of approximately 48 hours. During this time they can be handled, and transfer to incubators can take place. Johnston (2002) believes that charr eggs should be moved as quick as possible after fertilization, as his experience is higher mortality rate when using the other method. The most important is perhaps to treat the eggs with great care at any time of transfer. Regardless of transfer time, the eggs should be disinfected before or shortly after incubation starts. To read a protocol on buffodine disinfection, click [here](#). The disinfection shall remove bacteria that follow the eggs from the female fish they originated. These may not be infectious, but still have negative effect on the chemical equilibrium and exchange between the egg and the environment. Some viral or bacterial pathogens may be present on the egg surface (e.g. IPNV, IHNV and VHS), and although disinfection does not eliminate the pathogens completely it may reduce chances of infection significantly.

Different types of incubators are used for charr eggs. To look at some of the most used types, click [here](#). Dependent on incubator type, the eggs should lay in only one layer when possible. This eases work such as dead egg removal and improves general control of the eggs. The basic needs of the eggs during incubation is oxygen and to get rid of metabolites. In order to provide this, it is useful to know the amount of eggs in each incubator. To find out you can count the eggs using a rack that counts a high number of eggs at a time (i.e. 200 or 500). You can also measure the water displacement of a known number of eggs to get the volume, that later can be used to for back calculation of number of eggs on a number to volume ratio. The **oxygen** consumption of charr eggs is dependent on temperature but generally very low. Often is 1 litre of water per litre of eggs used as a reference. The water flow should not disturb the eggs, but flow gently past them. Since the oxygen uptake of the eggs are dependent on a partial pressure difference over the eggshell to be effective, the oxygen content of the water should be kept close to 100%. Oxygen tension in the water outlet should not be allowed to drop below 95%.

Direct **sunlight** is detrimental to charr eggs, and must be avoided. Regular indoor illumination may also be harmful, so the eggs should be covered and kept in darkness throughout the whole embryonic development. Especially high energy light may damage the genetic material of the embryo. Soft red light is less harmless and should be used when working with the eggs.

Temperature affects the embryonic development. It is common to express temperature dependent development in terms of accumulated temperature units (ATU). ATU is calculated by multiplying number of days in a period with average temperature during the period in question. The correlation between ATU and development is however not straight forward, as the temperature times days is reduced at low temperature, even though the number of days may increase. At 1 degree Celsius, 171 ATU (and days) elapse from spawn to hatch, whereas this increases to about 440 ATU at 6 degrees (but number of days is reduced to 73). Arctic

charr eggs are vulnerable towards high temperature. As a general rule temperature should be between 6 and 8 degrees Celsius. Johnston (2002) reports of increased mortality rates for most strains at temperatures above 7 degrees Celsius. The eggs are most vulnerable during the first period of development. After organogenesis (approx. 100 ATU) temperature may be increased to 8 degrees Celsius, but at no time exceed 10 degrees. Robbins with coworkers (1990) and Stickney (1990) presented data on temperature effects on egg development, and these are given in the figure below.

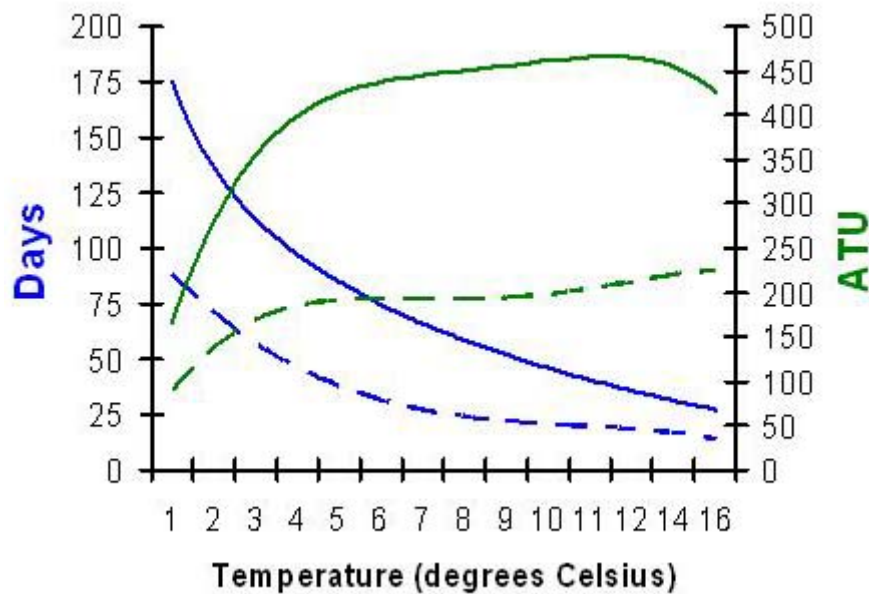


Figure 1. Effect of temperature on Arctic charr egg development from spawning and until the eyed stage (dotted line) and hatching (line). Time given as days (blue) and Accumulated Temperature Units (ATU, Green). Data from Robbins *et al.*, (1990) and Stickney (1990). Survival rates to hatch were between 85 and 90 % at temperatures below 7 degrees Celsius. At temperatures above 10 degrees, survival was 5 % or less.

Dead eggs should be removed as soon as possible from the incubators to avoid problems with bacterial and fungal infections. During the period from fertilization to the eyed stage, eggs are vulnerable to light and handling. Removal of dead eggs during this period should therefore be done very carefully. After the organogenesis have taken place and the eyes are visible eggs are relatively hardy and can be handled more easily without risking increased mortality. From the eyed stage and onwards unfertilized eggs should be removed, and the incubators rinsed thoroughly. The eggs can even be moved between incubators during cleaning. If the number of eggs per litre has not been done before, this is an appropriate time to do it. There are several ways of picking the eggs. You could use forceps and pick one egg at a time. This is effective if there are not too many dead eggs. A siphon is more effective than forceps since the operator need not lift each single egg, but simply move the siphon around the eggs letting the water drag the eggs out through the tube. This technique calls for a minimum of skills to avoid live eggs being picked by accident. A more sophisticated method to remove dead and unfertilized eggs is to use automated systems. Several types are available, but generally they are expensive and only large egg producers may find them cost effective. To ease identification of dead and unfertilized eggs, a procedure involving "shocking" the eggs are useful. Simply pour the eggs and water from one container to another, releasing them with a height of 50 - 100 cm. Eggs that are unfertilized or contain dead embryos will break due to this mechanical shock, and turn white as the yolk coagulates. In some cases fungal infections have already established, and in these cases it is better to remove the whole lump of eggs, for

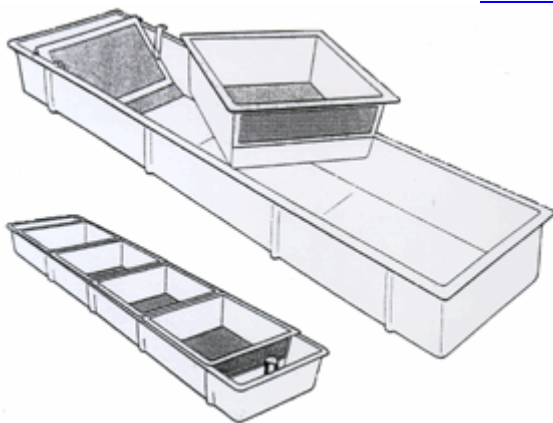
instance with a spoon. The operator needs to evaluate the need for treatment if the infections are severe. The equipment used must be changed or disinfected between incubators to avoid potential infections to spread. After dead eggs have been removed it is advised that all equipment used, together with clothing and working areas are cleaned and disinfected. To keep track of number of eggs, and to evaluate quality and health status of each egg batch, number of dead eggs picked should be carefully recorded.

Egg incubators

During embryonal development, the egg needs shelter and clean water. There are many different types of hatchery equipment built for this use, and below are four of the most common types. Click on the pictures to read more about them.



[Vertical stack incubator](#)



[Hatching troughs](#)



[Upwelling incubator](#)



Family hatcher