Norges veterinærhøgskole

Final report: Animal welfare aspects of marking farmed Atlantic salmon by the adipose fin clip method (FHF project # 900716)

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Sammendrag på norsk

Dyrevelferds-aspekter ved å fettfinne-merke oppdrettslaks (*Salmo salar* L.)

Hovedmålet med dette prosjektet var å beskrive den umiddelbare sårhelingsprosessen etter merking av Atlantisk laks med fettfinneklipping, og hva funnene betyr for vår vurdering av metoden når det gjelder fiskevelferd. Merkeforsøk ble gjennomført på lakseparr som ble holdt ved 4, 10 eller 14^{o}C. Det ble gjort 12 uttak av vevsprøver fra sårstedet de første 72 timene etter merking, og prøvene ble undersøkt med standard histologisk metode. Prøver fra frysemerket og fettfinnemerket fisk tatt ut over 4 måneder i et samarbeidende prosjekt ble også undersøkt.

Tiden fra merking til såret var lukket viste seg svært avhengig av vanntemperaturen. Hos fisk som ble holdt ved 4 °C var snittflaten dekket av et tynt lag med epidermale celler 12 timer etter merkingen, mens ved 10 °C eller 14 °C var sårflaten helt dekket av hudceller allerede ca. 4 timer etter merkingen. Epidermis hadde nådd tilnærmet normal tykkelse og differensiering etter ca. 6 timer (10^{o}C, 14^{o}C) respektive 18 timer (4^{o}C). Selv om lavere vanntemperatur resulterte i noe saktere lukking av såret, foregikk den innledende sårhelingsprosessen langt raskere enn vi hadde forventet. Denne raske sårlukkingen indikerer at laksefisk som merkes med fettfinneklipping kun opplever en svært kort periode med osmotisk påkjenning på grunn av at såret er åpent.

Effekten på den osmotiske balansen hos fisken ble ikke undersøkt i dette prosjektet, men kan antas minimal både på grunn av den beskjedne sårflaten og den korte varigheten. I forsøket fant vi ingen tegn til infeksjon eller inflammatorisk respons etter fettfinneklippingen. Som med alle fysiske merkemetoder må man likevel søke å begrense den mikrobielle belastningen i anestesibad og oppvåkningskar, og vi foreslår økt oppmerksomhet mot vannkvaliteten under og etter fysisk merking for å redusere muligheten for sekundærinfeksjoner. Merking med fettfinneklipping er ikke kostnadsfri, fordi metoden f. eks. vil øke tids- og bemanningsbehovet under vaksinasjon. Men vi tror det er en av de billigere og mest effektive merketeknikkene fordi det ikke er nødvendig med spesialisert utstyr.

Funnene i denne studien viser at fettfinnemerking kan gjennomføres uten å forårsake nevneverdig funksjonell påkjenning eller noen varig mén for fisken. Dermed tilsvarer metoden det som kalles «humane merkemetoder», slik det for eksempel nevnes i den europeiske konvensjon om beskyttelse av virveldyr som brukes til eksperimenter og andre vitenskapelige formål. Metoden er derfor etter vår mening godt egnet for rutinemessig merking av oppdrettslaks, forutsatt at den gjennomført på en kyndig måte og under velkontrollerte forhold.

Summary

The primary aim of this project was to identify and describe wound closure and healing processes following routine marking using 100% adipose fin clipping, and determine what impact this may have on overall fish welfare. A trial was conducted on salmon parr held at 4, 10 or 14^{o}C. Tissue samples from the wound site were obtained on 12 time points during the first 72 hours after marking, and the samples were examined by standard histological methods. Samples from freeze branded and adipose fin clipped fish taken over 4 months in a collaborative project were also examined.

The wound closure rate following adipose fin clipping was highly dependent on water temperature. In fish held at a constant 4 °C, a thin epidermal cell layer had fully closed the wounds 12h post fin clipping, whereas fish held at 10 °C or 14 °C had an epithelial layer fully closing the wounds 4h post fin clipping. The epidermis had reached close to normal thickness and cellular differentiation after ca. 6h (10^{o}C, 14^{o}C) or 18h (4^{o}C). Though lower water temperatures resulted in slower wound closure, it still progressed at a much higher rate than initially expected. This swift rate of wound closure indicates that fish marked using adipose fin clipping experiences only a short period of osmotic stress due to the presence of an open wound.

The effect on the osmotic balance of adipose fin clipped fish was not investigated, but may be viewed as minimal due to the relatively small wound area and the short duration. We found near to no signs of infection or inflammatory response following fin clipping. As with any marking method care must be taken to limit the microbiological load present in the anaesthesia bath and wakeup tank, and suggest increased vigilance regarding water quality during and after marking to reduce any possibility of secondary infections. It is acknowledged that adipose fin clipping is not cost-free and will increase the time and manpower needed during times of vaccination. However we believe it is one of the cheaper and more efficient marking techniques considering the fact that no specialised equipment is needed.

Considering these findings we suggest that this marking method can be conducted without causing notable impairment or lasting harm to the fish. Thus it constitutes a humane method, as defined in the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes, well suitable for batch marking large numbers of farmed Atlantic salmon if conducted in well controlled and maintained conditions.

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Introduction

To reduce and possibly avoid any notable negative effects of escapees on populations of wild fish or other natural fauna is a long-term strategy to secure the sustainability of Norwegian aquaculture. Together with the control of sea lice, the control of escapes has therefore been declared top priority by the organisation of Norwegian fish farming enterprises, the Norwegian Seafood Federation (FHL). Numerous measures to achieve this goal are being promoted in the short and medium term. As part of these efforts, there is a wish to establish simple and cost-effective methods to discriminate farm escapees from wild salmon being caught in fjords or rivers, and various techniques for marking the farmed fish population has been discussed. The identification of farmed fish with visible marks that can be readily recognised by laymen is of particular interest, because it would allow the re-release of unmarked (wild) fish while marked individuals (escapees) can be killed after capture. External markings would also support the management of prospective broodfish in fish ladders and traps, and thereby improve salmon conservation and stock enhancement efforts.

The current project sets out to perform an in-depth animal welfare assessment of one of the simplest and most widely employed method for marking salmonids, namely the adipose fin clip. This marking technique is easily performed on anaesthetised fish at any time from the parr stage, using a sharp straight or curved scissor or possibly automatic during vaccination. The adipose fin clip lends itself well for marking concurrently with injection vaccination, to which the entire Norwegian farmed salmon year classes are already subjected. In order to make such a marking technique mandatory on industry-wide scale, however, the animal welfare aspects of the procedure should be thoroughly scrutinised.

Various techniques for marking or tagging salmonids and other finfish have been comprehensively reviewed by Nielsen (1992). Here he discusses in depth the pros and cons of external tags (Floy tags or similar), external marks (fin clipping, branding, and pigment marks), internal tags (coded wire tags CWT; passive integrated transponder PIT tags, and visible implant tags), as well as a number of natural marks (otolith or scale analysis, morphometrics etc.) and chemical marking techniques (fluorescent chemical, calcein). Each marking technique has its own set of constraints with arguably the most important being retention time (Dietrich and

Cunjak, 2006). When conducting a long term study it is imperative that the tags remain visible throughout the study, otherwise the results may be negatively affected by inaccurate recording. Yet certain techniques may reduce survival if incorrectly applied or if the fish are too young, therefore it is imperative that all personnel are well trained prior to conducting the procedure (Wydowski and Emery, 1983). However, the wish to discriminate between two groups only (escaped farmed individuals from the truly wild fish) differs from the diverse needs of wild fish research and management, that forms the background for most of the work reviewed in Nielsen (1992).

Among the important limitations of using scale analyses (Lund and Hansen, 1991) or fluorescent chemical marking on otolith or scales are that these methods cannot provide immediate discrimination in the field, but require samples to be submitted to specialised laboratories for analysis. This is partly true also for discrimination based on morphology characters such as body shape and the integrity of fins (Lund *et al*., 1989; Friedland *et al*., 1994) that unless assisted by additional techniques are believed prone to misclassification.

Due to the fact that virtually the entire Norwegian farmed Atlantic salmon population undergoes immunisation with multivalent, injectable vaccines, the intra-abdominal lesions and scars resulting from vaccination lends itself to distinguish farmed from unvaccinated wild salmon (Lund *et al*., 1997). This assessment may be performed in the field with the naked eye, but the fish has to be killed and opened making it unsuitable for situations where truly wild fish should be released after classification. Vaccination also produces antibody responses to several of the antigens that are ≥1000-fold higher than any natural infection (Midtlyng, pers. obs.) but despite the fact that blood samples can be drawn without sacrificing the fish, the samples must be submitted to specialised laboratories for analysis, a procedure which may take several days.

We know that the adipose fin clip represents a simple and cost-effective method for marking the entire farmed Atlantic salmon population, and will allow the easy and immediate recognition of unmarked (wild) live fish with the naked eye. The adipose fin clip is generally held to cause no or minor negative effects on fish survival (Gunnes and Refstie, 1988), even in the wild (Johnsen and Ugedal, 1988); a finding that has been confirmed during extensive experimental and field trials with vaccination of Atlantic salmon over many years (Midtlyng, pers. obs.).

Mandatory fin clip is therefore an obvious candidate to allow the easy identification of farmed salmon escapees if caught in fjords or rivers.

However, when prescribing external markings by law, and when subjecting entire year classes (>200 mill individuals) of farmed Atlantic salmon to adipose fin clip, a thorough scientific assessment of the animal welfare aspects of the procedure needs to be undertaken.

Project elements and aims

The current project sets out to document its essential animal welfare aspects in this species with the following elements:

Wound healing process following removal of the adipose fin

Previous studies on wound healing in rainbow trout being held at 15 °C has shown that following an incision through the skin into the muscular layers, closure of the wound by the epidermal mucus layer was rapid (<3 days) and that the healing process was more or less completed after 21 days (Wahli *et al*., 2003). A similar experiment performed in Atlantic salmon parr held at 12 °C yielded essentially similar results (P. Midtlyng, confidential project report). As the area of epidermal damage after adipose fin clip is likely very small, we aimed to assess in detail the time needed for epidermal cells to migrate and to close the wound. In particular, the temperature dependence of this and further wound healing processes were subject to assessment by using microscopic methods and relevant specific stains. For this purpose, we established collaborative relations with the Fish and Wildlife Health Laboratory at the University of Berne, Switzerland, the group currently most experienced with skin histology of fish.

Adipose fin clipping is a frequently used technique to mark salmonids in both research and culture environments (Vander Haegen *et al.* 2005). This method is time and cost efficient, as very little equipment is needed and clipping may be performed in combination with other management practices such as vaccination. The scientific interest in this method has increased as the effect on the fish is not fully understood. This is complicated by the fact that the exact role of the adipose fin has not yet been identified. Reimchen and Temple (2004) conducted a study to identify the function of the adipose fin. They determined that it may have a role in controlling vortices before they reach the caudal fin, or it may function as a pre-caudal sensor of turbulent

flow. This project was conducted in order to identify the wound closure rate and processes following adipose fin clipping which in turn may aid with determining the effect clipping may have on the fish welfare.

In addition to adipose fin clipping we aimed to describe the initial wound closure and healing processes following freeze branding and visible implant elastomers. Freeze branding is conducted by exposing a stainless steel brand to liquid nitrogen, once deemed cold enough the brand is placed against the fish (usually a designated flank) for a short time, usually 1-3 seconds (Chart and Bergersen; 1988, Evrard, 2005). The advantage of this technique is that the necessary equipment is relatively cheap and therefore the cost per fish is relatively low. It also allows for a variety of symbols to be used to mark the fish, thus providing opportunities for multiple group marking. However, the marks may fade as the fish grows and the time needed to mark large batches of fish may be an issue (V. Puvanendran, pers. comm.). Visible implant elastomer tagging (VIE) is conducted by subcutaneously injecting a fluorescent elastomer in the designated area of the body surface, often just below the dorsal fin or in the operculum near the eye (FitzGerald *et al.* 2004). The tag is visible without the need of additional equipment or sacrificing the fish; ultraviolet light makes them glow and results in easy identification (V. Puvanendran, pers. comm.). However, a long term study conducted by FitzGerald *et al.* (2004) resulted in mixed results with some loss in visibility being recorded after 17 months.

WP1: Project management overview

A full overview of the progress for each work package over the course of the project has been submitted to the sponsor. This project was conducted by the Norwegian School of Veterinary Science (NVH) with the primary researcher being Dr Melanie Andrews (NVH) additional assistance was provided by Dr Paul J Midtlyng, Prof. Eystein Skjerve and Dr Arnfinn Aunsmo from the NVH. The experiment comprising WP 2.3 was conducted at the VESO Vikan facility by Dr Anne Ramstad and her research team. Preparation and analysis of the histology samples was conducted by Dr Thomas Wahli and Dr Heike Schmidt-Posthaus at the Vetsuisse Institute, University of Bern. Interpretation of the results was conducted in collaboration between Dr Melanie Andrews and researchers from the Vetsuisse Institute.

WP2: Epidermal healing processes following physical marking of Atlantic salmon pre-smolts

Materials and methods for all experiments in WP2

Experimental setup

This work package comprised of three experiments which shared some elements, a description of all three is provided below.

Work package (WP) 2.1 was conducted to determine whether the proposed experimental plan and sampling timeline were feasible. A total of 36 Atlantic salmon parr (mean 16,6g; range 7-30g) were randomly selected and placed in a 250 L (water temperature 9-11 °C) tank to acclimate. Once acclimated they were placed in a small anaesthetic bath with 15 µg/L Aqui-S® until sufficiently anaesthetised, after which they were 100% adipose fin clipped and returned to the experimental tank (see Figure 1, Section 3.2). Fin clipping was conducted using a sharp and sterilised pair of scissors. Observations were made to ensure that all fish recovered from the anaesthesia. Sampling was conducted over 12 time points (0, 2, 4, 6, 12, 18, 24, 30, 36, 48, 60,

and 72 h post clipping) over a 72 h period. Three fish were sampled at each time point with samples of the wound area preserved immediately in 10% neutral buffered formalin and sent to the University of Bern for histology processing and analysis.

The second experiment (WP 2.2) was conducted by Nofima, Tromsø from which we obtained samples taken over a 3 week period. A total of 135 Atlantic salmon (mean 11,3g; range 9,7-12,8g) were randomly selected and placed in a 450 L tank. Following the acclimation period the fish were marked, the methods used in this experiment were 100% adipose fin clipping (25 fish), 75% adipose fin clipping (25 fish), visible implant elastomer (VIE) on the operculum (25 fish), and freeze branding below the dorsal fin (25 fish), and 10 unmarked control fish. Five fish from each marking group were sampled at five time points (0h, 12-24h, 3 days, 7 days, 14 days, 21 days, and 4 months) post marking. Sampling was conducted by Nofima staff, placed immediately in 10% neutral buffered formalin and sent to the NVH in Oslo. The samples were then sent to the University of Bern for histology processing and analysis.

The main project experiment (WP 2.3) was conducted at the VESO Vikan facility, near Namsos using the experimental setup established in WP 2.1. During the experiment 204 Atlantic salmon smolts (mean 36g; range 27,7-45,3g) were split into three 450 L tanks (n=66) with each tank set at a constant water temperature of 4, 10, and 14 ºC. An additional 6 fish were placed in the 10 ºC tank to be the 0 h samples for all temperature groups. The fish were acclimated for one week and starved for 24 h prior to commencing the experiment. At commencement all fish from the 4 ºC group were placed in an anaesthetic bath (15 µg/L Aqui-S®) using water from the holding tank. Once sufficiently anaesthetised, the adipose fins were clipped (100%) using scissors and then returned to the holding tank. This was repeated for the 10 ºC and 14 ºC groups, with all groups being adipose clipped within a 30 min period. Following fin clipping all fish were observed to ensure complete recovery from the anaesthetic. Sampling was conducted over 12 time points over a 72 h period, similar to the pilot study yet with 6 fish sampled from each group at each time point. However, only 6 fish were sampled at 0 h controls to be used for all groups. Samples were carefully taken so as not to disturb the wound area and placed immediately in 10% neutral buffered formalin and sent to the University of Bern for histology sectioning and analysis.

Histology analysis and scoring

Histology slides were prepared at the University of Bern's Vetsuisse Pathology section. Using a transverse cut through the wound area, a single slide was prepared for each fish and stained using haematoxylin and eosin (HE) stain. Each histology slide was examined and scored using the score sheet provided Table 1 which was adapted from Wahli *et al*., (2003).

A total of 11 parameters regarding the form of the epidermis and dermis were measured for each sample with all parameters being scored using a linear scale ranging from 0 (abnormal) to 30 (normal). Parameters 1 to 7 cover the following aspects of the epidermal layer (Table 1); (1) General epidermal structure, all cell layers are present with all cells in their normal form; (2) Epidermal thickness is difficult to determine as normally is relative, however in this case we found that uniform thickness across the wound area is normal; (3) The basal cell layer should comprise cuboidal to columnar cells; (4) The cell layer between the inner basal and superficial cell layers consists of round cells which are round to cuboidal in form; (5) The outermost cell layer is the superficial cell layer comprising elongated and flattened cells; (6) Mucous cells should be scattered throughout the epidermis; (7) Presence of infiltration granulocytes, lymphocytes or macrophages within the wound area. Parameters 8 to 11 cover the following aspects of the dermal layer (Table 1); (8) General dermal structure, all cell layers are present with all cells in their normal form; (9) Presence of necrosis, oedema, cell debris or non-eosinophilic staining amorphous material; (10) Presence of infiltration granulocytes, lymphocytes or macrophages within the wound area; (11) A continuous layer of pigment cells immediately below the basement membrane.

All histology slides were examined at 200x magnification using light microscopy setup and all scoring was conducted blindly by a single histopathologist in order to ensure consistency and eliminate bias. In addition, comparisons were made at each time point between the three temperature groups to determine whether there was a difference in the rate of wound closure and initial healing processes. A summary is included in Tables 3 and 4 with graphical representations provided in Figures 4 and 5.

Statistical analysis of the scoring sheets was not easily done due to the unequal numbers of samples for each time point and temperature. Equal numbers of samples were taken from each water temperature group at each time point. However, scoring could not be completed for each sample due to some samples being deemed unusable. It was found that, possibly due to irregularity in sample sizes, the histology technicians missed the area of interest on some samples. Examination of the histology samples clearly illustrates the wound closure process; however, the mean values for each observation in the score sheet (Table 1) have been graphically represented in the results section for work package 2,3.

Table 1. Scoring sheet used to determine the presence and appearance of the cells comprising the epidermis and dermis following adipose fin clipping of Atlantic salmon (*Salmo salar* L.), with a score of 0 being absent/abnormal and 30 being present/normal.

	Nr.	Parameter	Description	Scoring
Epidermis	1	Structure	Normal epidermal structure consisting: basal cell layer, several layers of rounded/cuboid cells with diffusely distributed mucous cells, and a superficial layer of flattened cells	0 = abnormal/non-uniform; 30 = normal; all layers present in their normal form
	2	Thickness	Uniform thickness of epidermis	0 = too thin/thick; 30 = normal
	3	Basal cells	Basal cell layer adjacent to basement membrane; cells cuboidal to columnar	0 = abnormal/non-uniform; 30 = normal density and shape
	4	Round cells	Located between basal cell layer and superficial epithelial cell layer; cells round to cuboidal	0 = abnormal/non-uniform; 30 = normal density and shape
	5	Superficial cell layer	Outermost cell layer; cells elongated, flattened cells	0 = abnormal/non-uniform; 30 = normal density and shape
	6	Mucous cells	Mucous cells present throughout the epidermis	0 = abnormal distribution; 30 = normal density and distribution
	7	Infiltration	Presence of infiltration granulocytes, lymphocytes, macrophages	0 = high infiltration; 30 = no infiltration
Dermis	8	Structure	Normal dermal structure consisting: basement membrane, pigment cell layer, stratum spongiosum (no scales present), stratum compactum, and a hypodermal layer	0 = abnormal/non-uniform; 30 = normal; all layers present in their normal form
	9	Cell debris	Presence of necrotic cells, oedema, cell debris and non-cellular eosinophilic staining amorphous material	0 = high number present; 30 = none present
	10	Infiltration	Infiltration granulocytes, lymphocytes, macrophages	0 = high infiltration; 30 = no infiltration
	11	Pigment cells	Cell layer immediately below basal membrane	0 = abnormal/non-uniform; 30 = normal density and shape

WP 2.1: Pilot experiment for the adaptation of tissue fixation techniques and sampling sequence

This pilot experiment was conducted to test whether the proposed experimental protocol was appropriate for the large scale experiment to be conducted in WP 2.3. The following are the results, discussing aspects we determined to be of most importance when conducting further large scale experiments:

- *Number of participants:* We found that the sampling schedule made it difficult for a single researcher to complete. This is particularly due to the frequent sampling over a condensed time period. We therefore ensured that the full scale experiments had at least 2 participants to conduct the sampling throughout the course of the experiment. This then eliminated the chance of errors occurring due to fatigue or inattention.
- *Experimental fish quality:* The fish used in this pilot study were sourced from mixed batches which resulted in a wide size range (7-30g). This was not ideal as the size of the adipose fins ranged so much that the healing rates varied. Therefore, in future experiments it was necessary to source from a single group of fish which would reduce such variability.
- *Sampling protocol:* The sampling protocol used during this study provided samples at the appropriate frequency to properly describe the initial wound closure process following adipose fin clipping. We ensured that the wound area was not damaged by removing a large section of tissue surrounding the adipose fin (Figure 1A, B).
- *Preparation of samples:* It was possible to include the entire wound area in the histological analysis by cutting the wound area in half (Figure 1B) and placing the entire sample in an histology cassette (Figure 2). Care was taken to ensure that the samples would not shift when in the cassette. The samples were sent to the University of Bern to be prepared and examined by their pathology department. They reported that the samples arrived in excellent condition, thus the preparation and postage section of the protocol proved acceptable.

- *Histological analysis:* Analysis of the samples using histological techniques illustrated that the overall planning and execution of the experiments was adequate to achieve the results we had hoped for. However they noted that the large size range of the fish used in this experiment resulted in inconsistent healing data. Therefore it was determined that the full scale experiments should use fish from a single production, thus reducing the variability.

Figure 1. A representation of the sampling area *in situ* (A), an illustration of the excised area (B) with the wound (red zone) and the histological incision (blue line) is included.

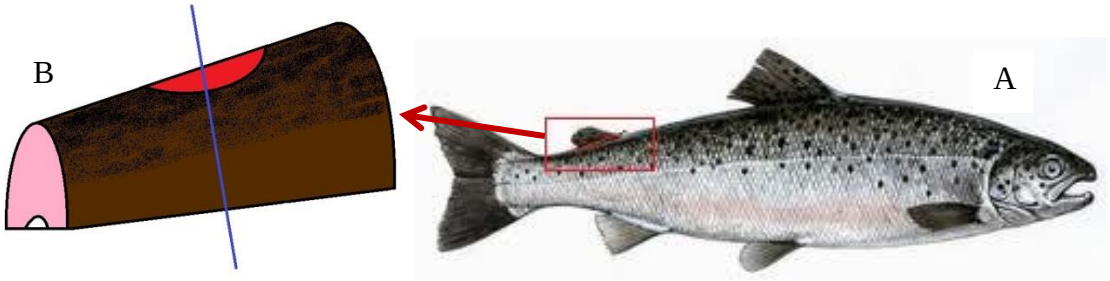
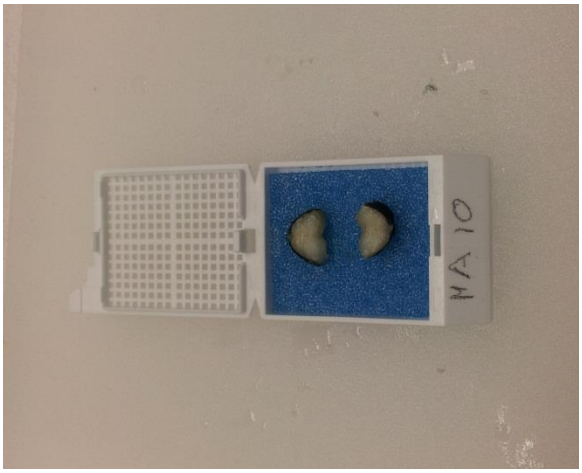


Figure 2. Illustrating placement of the adipose fin clip samples in histology cassettes in preparation for histological analysis.



WP 2.2: Histology comparison of three marking methods employed in the Nofima trial

This work package aimed to determine the wound closure and healing rates in samples from the Nofima project, employing three marking techniques; 100% adipose fin clipping, freeze branding, and insertion of visible implant elastomers (VIE). However, we were included in this experiment directly before it commenced. This left us little time to adequately plan which sampling methods to use that will yield the best histological results. This resulted in only the 100% adipose fin clipping and freeze branding samples being suitable for examination using histological techniques.

All fish exhibited complete wound closure between 12 and 24 hours following 100% adipose fin clipping, with relatively normal epidermal and dermal scores from this time point onwards (Table 2). The fish were held at low temperatures ranging between 4 and 6 °C and exhibited similar healing rates to that observed during WP 2.3. As this experiment was conducted over a relatively long time period they experienced regular husbandry practices, including feeding, tank cleaning and general health observations when conducting sampling. Despite these external factors the healing rate of adipose fin clipped fish did not differ significantly from what we observed in the more well-controlled experiment (see below).

Table 2. Summary of the mean results for each time point following 100% adipose fin clipping using the scoring sheet provided in Table 1.

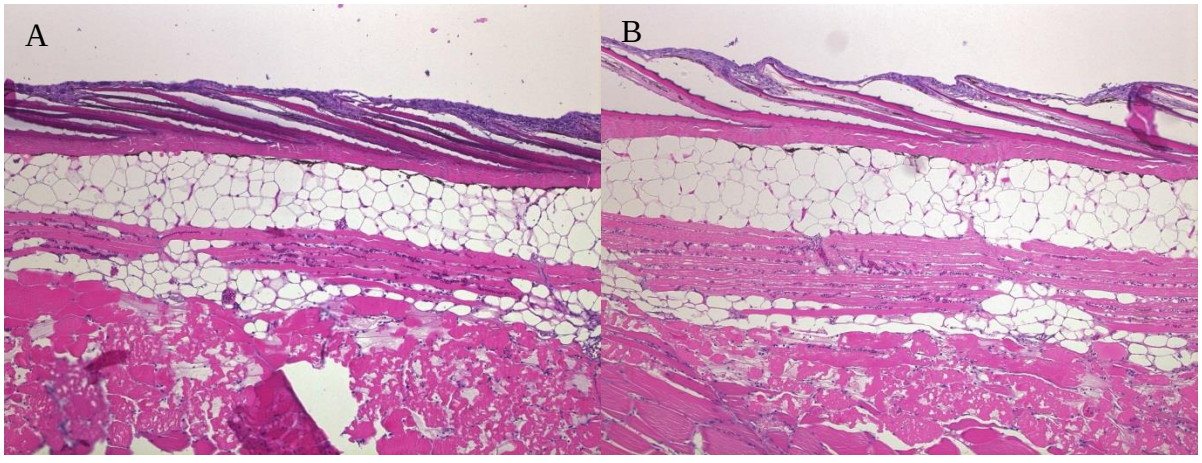
Time point	Structure (Epidermis)	Thickness (Epidermis)	Basal cells (Epidermis)	Round cells (Epidermis)	Superficial cell layer (Epidermis)	Mucus (Epidermis)	Infiltration (Epidermis)	Structure (Dermis)	Cell debris (Dermis)	Infiltration (Dermis)	Pigment cells
0h	0	0	0	0	0	0	30	16	28	30	0
12-24h	26	29	23	24	27	29	28	28	25	26	20
3 days	28	28	27	27	28	27	29	27	27	28	24
1 week	30	30	30	29	29	29	29	30	30	30	30
2 weeks	27	27	24	25	26	27	26	26	26	28	20
3 weeks	29	29	28	28	30	30	28	30	30	30	28
4 months	27	28	26	26	29	27	28	28	27	29	21
Control	29	29	28	29	29	28	29	29	28	29	27

The results obtained from freeze branded group were not as clear as that from the adipose fin clipped group. In comparison to the adipose fin clipped fish at 0h (Table 2), where the entire epidermis and dermis was removed, freeze branding did not completely remove the epidermis and the dermis is only slightly affected. Healing rates differed slightly between fish sampled at the same time points (Figure 3); this indicates difference in depth of the freeze brand which may be due to inconsistent exposure times. We may surmise that this inconsistency caused the deviation in score which may be seen in Table 3. The fact that the epidermis was not completely healed 2 weeks following freeze branding, and that inconsistent depth of branding occurred, leads us to conclude that this method is not entirely suitable for marking large groups of fish.

Table 3. Summary of the mean results for each time point following freeze branding using the scoring sheet provided in Table 1.

Time point	Structure (Epidermis)	Thickness (Epidermis)	Basal cells (Epidermis)	Round cells (Epidermis)	Superficial cell layer (Epidermis)	Mucus (Epidermis)	Infiltration (Epidermis)	Structure (Dermis)	Cell debris (Dermis)	Infiltration (Dermis)	Pigment cells
0h	12	12	10	10	12	11	25	20	26	26	20
12-24h	17	16	12	12	16	12	28	30	30	30	30
3 days	22	22	19	19	21	19	28	30	30	30	30
1 week	6	8	5	2	6	4	28	28	29	30	29
2 weeks	21	20	19	18	21	21	28	30	30	30	30
Control	30	30	30	30	30	30	30	30	30	30	30

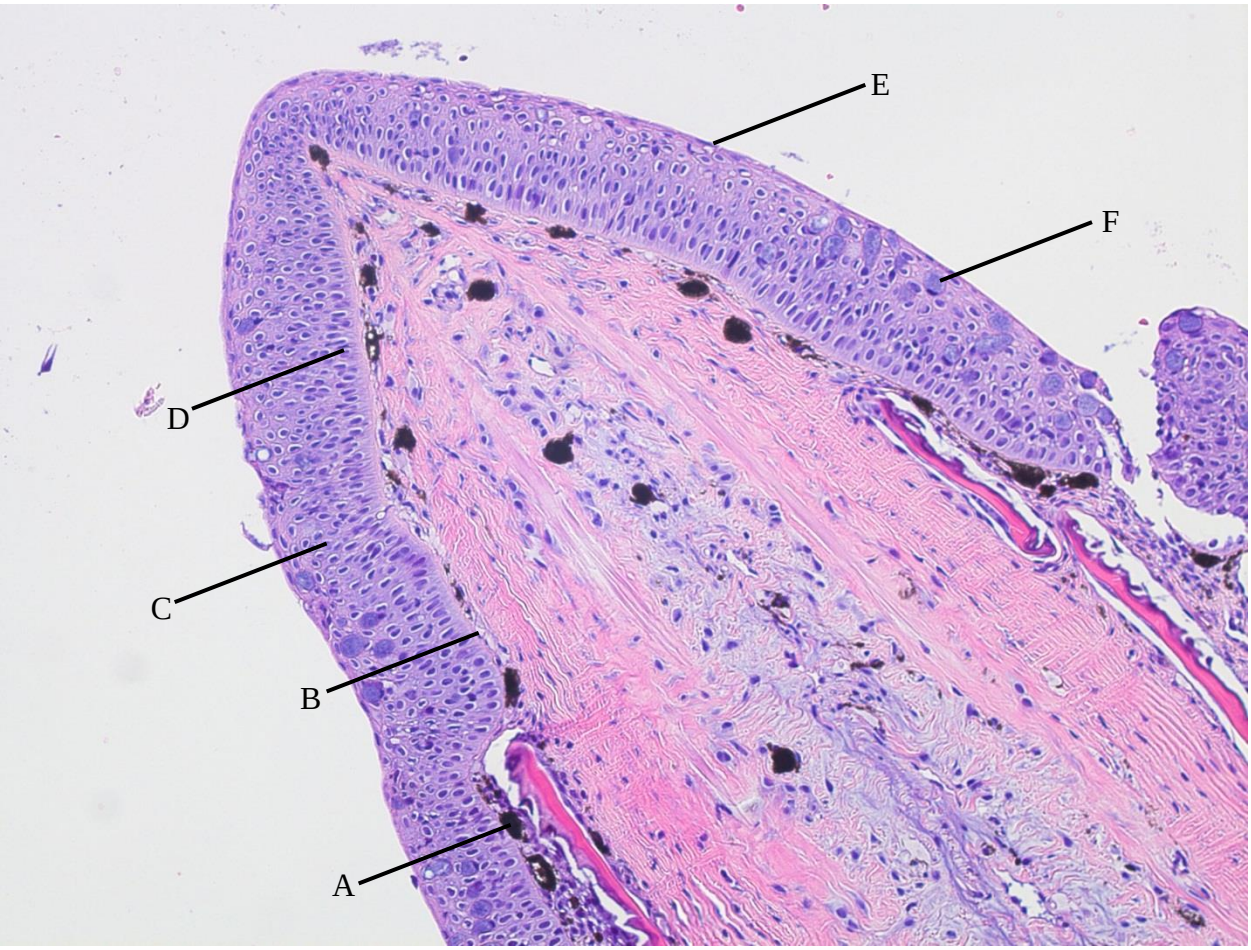
Figure 3. Example of varying rates of re-epithelisation 1 week after freeze branding, (A) has a markedly thicker epidermis than (B).



WP 2.3: Time course and temperature dependence of epidermal and cutaneous repair

This work package was conducted using the methods described, modified using the results described in previous sections. The six primary features observed and scored at each time point have been detailed in Figure 4. In normal adipose fins the pigment cells are distributed just below the basement membrane throughout the adipose fin. The basal cells should present as a row of uniform cuboidal cells located alongside the basement membrane. The basement membrane is a thin layer of connective tissue that supports and attaches the epithelium to the adipose fin, whereas the mucous cells are usually distributed throughout the epidermis.

Figure 4. Histology section detailing the cell groups present in the adipose fin of the Atlantic salmon; including (A) pigment cells, (B) basement membrane, (C) round cells, (D) basal cells, (E) superficial cell layer, (F) mucous cells.



The round cells make up the central region of the epidermis and present as square/round cells which fit well together with no intercellular spaces. The superficial layer is the outermost epithelial layer which consists of evenly distributed flattened epithelial cells that effectively seals in the underlying epithelial layers.

The 0h samples were conducted on the 10 °C as the fish were immediately sampled following clipping resulting in the water temperature having no effect on the fin and there has been no time for commencement of healing processes. Figure 7A illustrates a clear incision site with the only cellular reaction being the formation of an area of oedema.

The primary cellular reaction observed across all three water temperatures is the formation of an oedematous region in the centre of the adipose fin (Figs. 7B-D). As no reformation of the epidermal layers was observed the mean scores were similar for all three temperature groups (Table 4; Figs. 5, 6).

The first major change between the temperature groups was observed at 4h post-clipping. No re-epithelisation was observed in the 4 °C group (Fig. 8A, Table 4); this is reflected in the scoring sheet. The first signs of re-epithelisation were observed at this time point in both the 10 °C (Fig. 8B) and 14 °C (Fig. 8C). Here a thin epithelial layer has formed across the wound area; however it is not possible to distinguish between the different cell groups (Table 4). This inability to distinguish resulted in similar mean results from the scoring sheet (Figs. 5, 6).

No re-epithelisation was observed in the 4 °C fish 6h post-clipping (Figs. 5, 8D); however the epidermis continues to develop in both the 10 °C (Fig. 8E) and 14 °C (Fig. 8F). In both of these groups it is possible to distinguish between the cell types (Table 4), however the epidermis is not uniform with vacuous regions present (Fig. 8F).

The first evidence of re-epithelisation was observed in the 4 °C fish (Figs. 5, 9A) 12h post-clipping. Here the epidermis is very thin and stretched and the different cell types are not discernible (Table 4). The epidermis continues to thicken in both the 10 °C (Fig. 9B) and 14 °C (Fig 9C) fish. All cell types, except the pigment cells, were distinguishable (Table 4). However, both present uneven epidermal thickness across the wound area as well as the presence of vacuous areas between the round cells.

At 18h post-clipping from all three water temperatures present similar epidermal thickness (Figs. 9D-F). However vacuous areas are present and mucous cells are not as thoroughly distributed as expected. At this stage all cell groups were distinguishable (Table 4).

By 24h post-clipping (Figs. 10A-C) mucous cells are present in greater numbers (Fig. 10A). Four of the main cell groups are distinguishable (Table 4), however vacuous areas are still present (Fig. 10C). In addition, the overall epidermal structure was similar for all three temperature groups (Table 4; Figs. 5, 6).

At 30h post-clipping all three temperature groups proceed to heal at the same rate (Figs. 10D-F); all cell types (excluding pigment cells) are visible at this stage (Figs. 5, 6). By 36h post-clipping (Figs. 11A-C) the mucous cells are more numerous throughout the epidermis (Fig. 11A), however vacuous areas (Fig. 11B) and compressed areas are still present (Fig. 11C). At 48h post-clipping (Figs. 11D-F) the vacuous areas are still present (Figs. 11E, F) and the epidermis has an uneven appearance (Fig 11D). This remains unchanged 60h post-clipping (Fig. 12A-C), and 72h post-clipping (Fig. 12D-F).

Table 4. Table illustrating the time points when the different cells and cell layers were observed for each temperature group following adipose fin clipping, with (×) indicating not present; (-) cells are present but not discernible between different cell types; (o) cells are present and distinguishable from other cell types.

Time	Tissue	Temperature (°C)			Time	Tissue	Temperature (°C)		
		4	10	14			4	10	14
0 h	Basal cell layer	×	×	×	24 h	Basal cell layer	o	o	o
	Round cell layer	×	×	×		Round cell layer	o	o	o
	Superficial cell layer	×	×	×		Superficial cell layer	o	o	o
	Mucous cells	×	×	×		Mucous cells	o	o	o
	Pigment cells	×	×	×		Pigment cells	×	×	×
2 h	Basal cell layer	×	×	×	30 h	Basal cell layer	o	o	o
	Round cell layer	×	×	×		Round cell layer	o	o	o
	Superficial cell layer	×	×	×		Superficial cell layer	o	o	o
	Mucous cells	×	×	×		Mucous cells	o	o	o
	Pigment cells	×	×	×		Pigment cells	×	×	×
4 h	Basal cell layer	×	-	-	36 h	Basal cell layer	o	o	o
	Round cell layer	×	-	-		Round cell layer	o	o	o
	Superficial cell layer	×	-	-		Superficial cell layer	o	o	o
	Mucous cells	×	×	×		Mucous cells	o	o	o
	Pigment cells	×	×	×		Pigment cells	×	×	×
6 h	Basal cell layer	×	o	o	48 h	Basal cell layer	o	o	o
	Round cell layer	×	-	o		Round cell layer	o	o	o
	Superficial cell layer	×	-	o		Superficial cell layer	o	o	o
	Mucous cells	×	o	o		Mucous cells	o	o	o
	Pigment cells	×	×	×		Pigment cells	×	×	×
12 h	Basal cell layer	-	o	o	60 h	Basal cell layer	o	o	o
	Round cell layer	-	o	o		Round cell layer	o	o	o
	Superficial cell layer	-	o	o		Superficial cell layer	o	o	o
	Mucous cells	×	o	o		Mucous cells	o	o	o
	Pigment cells	×	×	×		Pigment cells	×	×	×
18 h	Basal cell layer	o	o	o	72 h	Basal cell layer	o	o	o
	Round cell layer	o	o	o		Round cell layer	o	o	o
	Superficial cell layer	o	o	o		Superficial cell layer	o	o	o
	Mucous cells	o	o	o		Mucous cells	o	o	o
	Pigment cells	×	×	×		Pigment cells	×	×	×

Figure 5. Graphical representation of *Table 4* illustrating re-epithelisation of the epidermis over a 72h period at three water temperatures (4, 10, 14 °C).

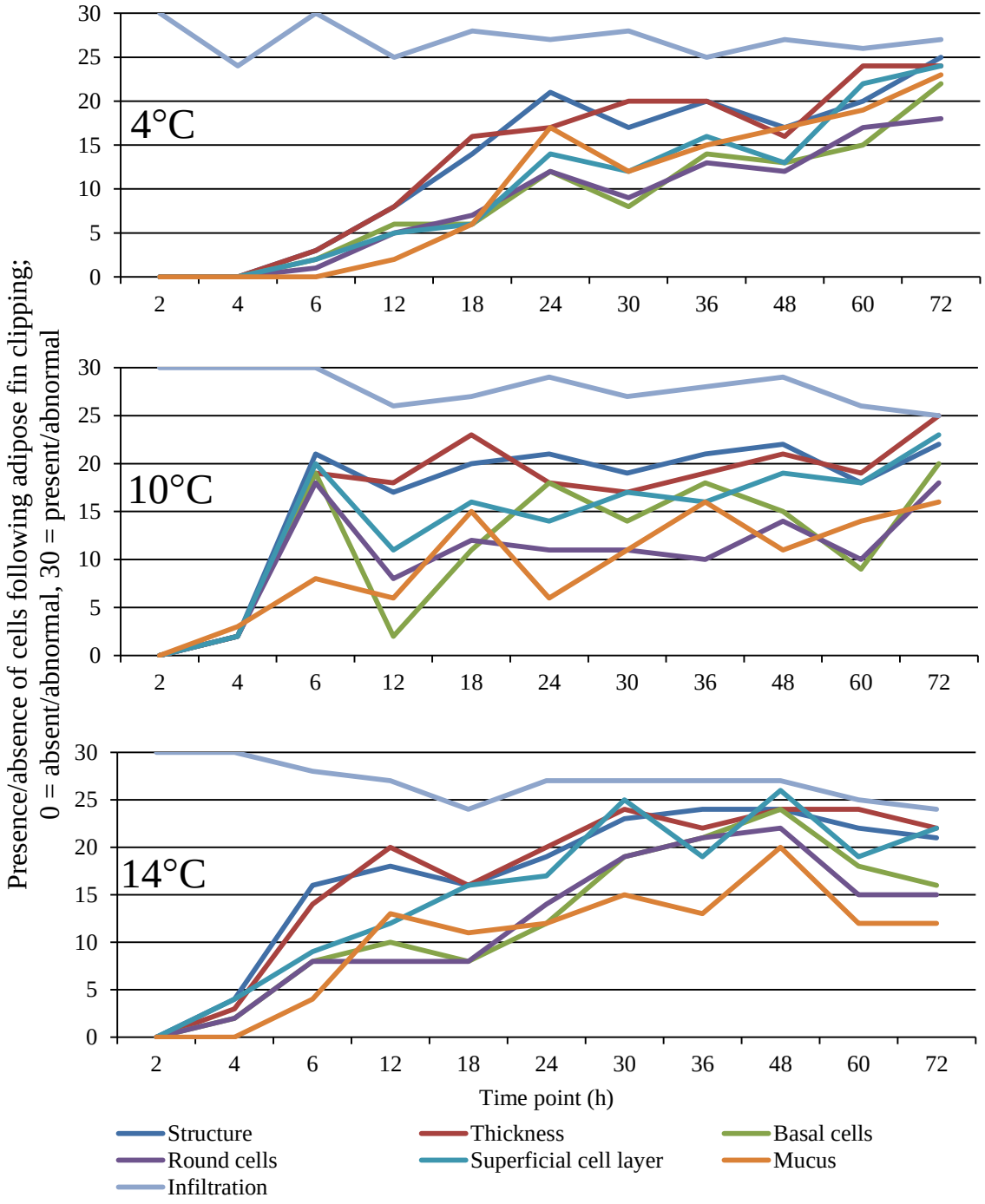


Figure 6. Graphical representation of *Table 4* illustrating re-epithelisation of the dermis over a 72h period at three water temperatures (4, 10, 14 °C).

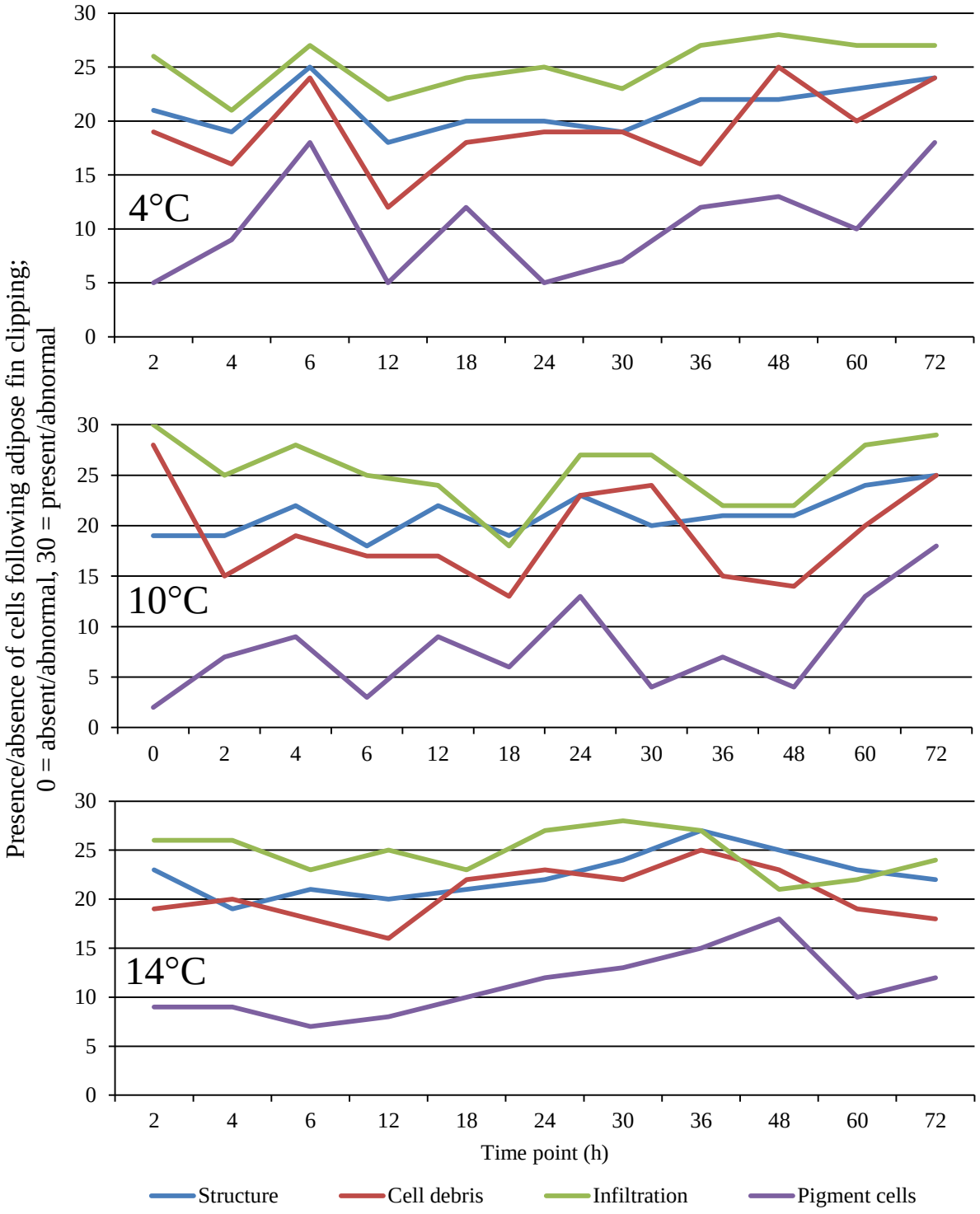
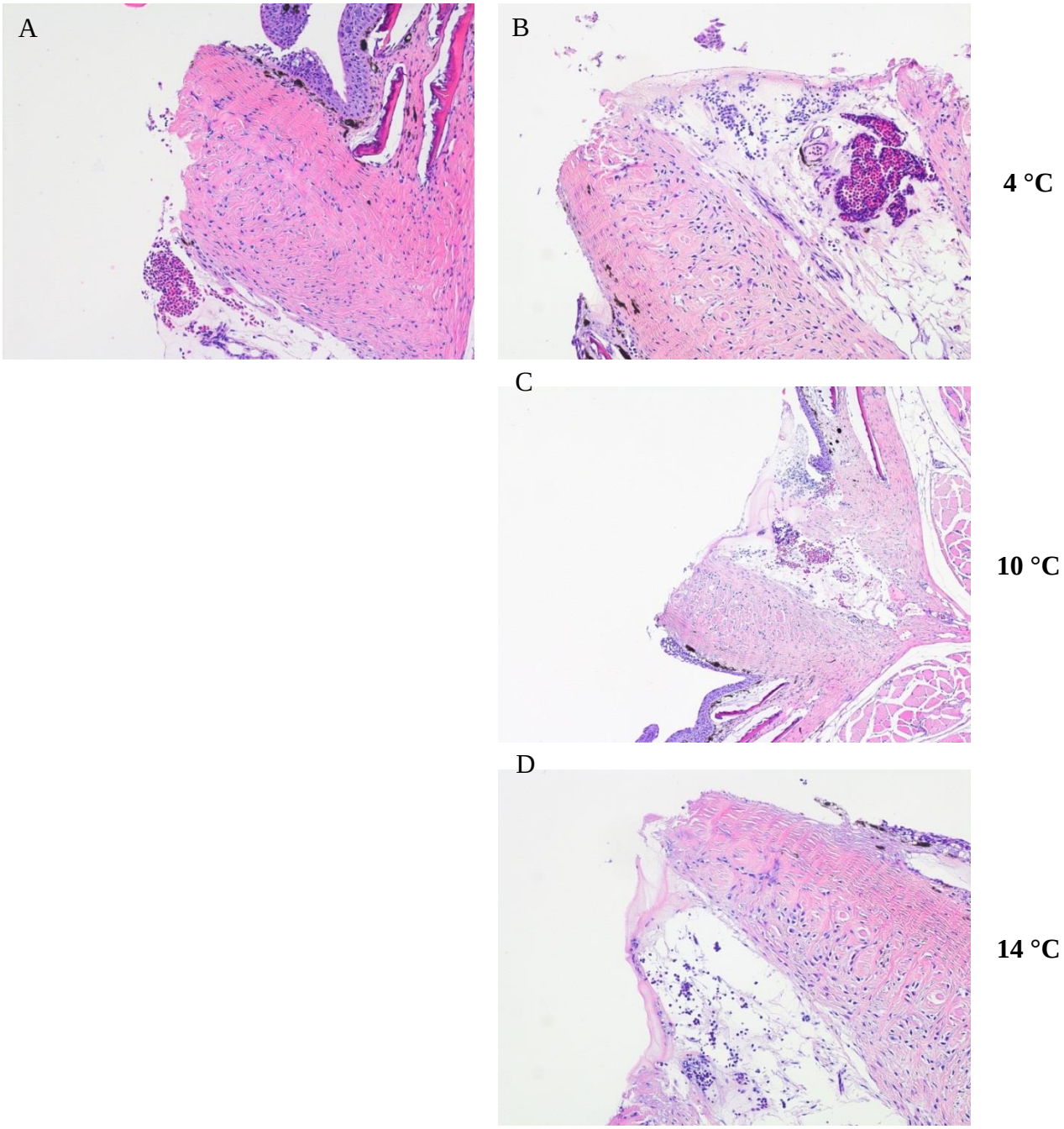


Figure 7. Illustrating wound closure and initial healing following adipose fin clipping; (A) 0 h post-clip; (B) 2 h post-clip, 4 °C; (C) 2 h post-clip, 10 °C; (D) 2 h post-clip, 14 °C.

0 hours post-clip **2 hours post-clip**



200x magnification

Figure 8. Illustrating wound closure and initial healing following adipose fin clipping; (A) 4h post-clip, 4 °C; (B) 4h post-clip, 10 °C; (C) 4h post-clip, 14 °C. (D) 6h post-clip, 4 °C; (E) 6h post-clip, 10 °C; (F) 6h post-clip, 14 °C.

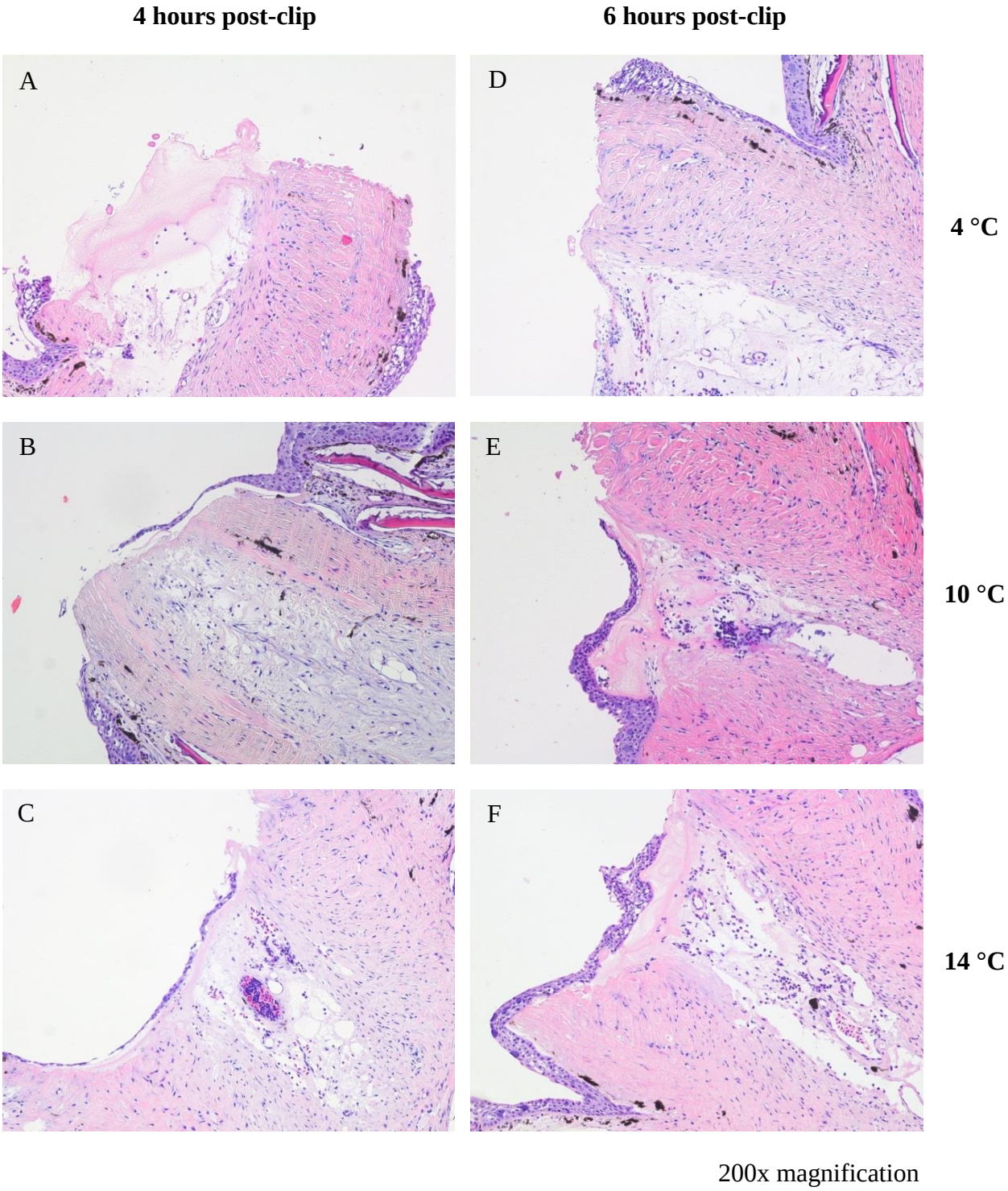


Figure 9. Illustrating wound closure and initial healing following adipose fin clipping; (A) 12h post-clip, 4 °C; (B) 12h post-clip, 10 °C; (C) 12h post-clip, 14 °C. (D) 18h post-clip, 4 °C; (E) 18h post-clip, 10 °C; (F) 18h post-clip, 14 °C.

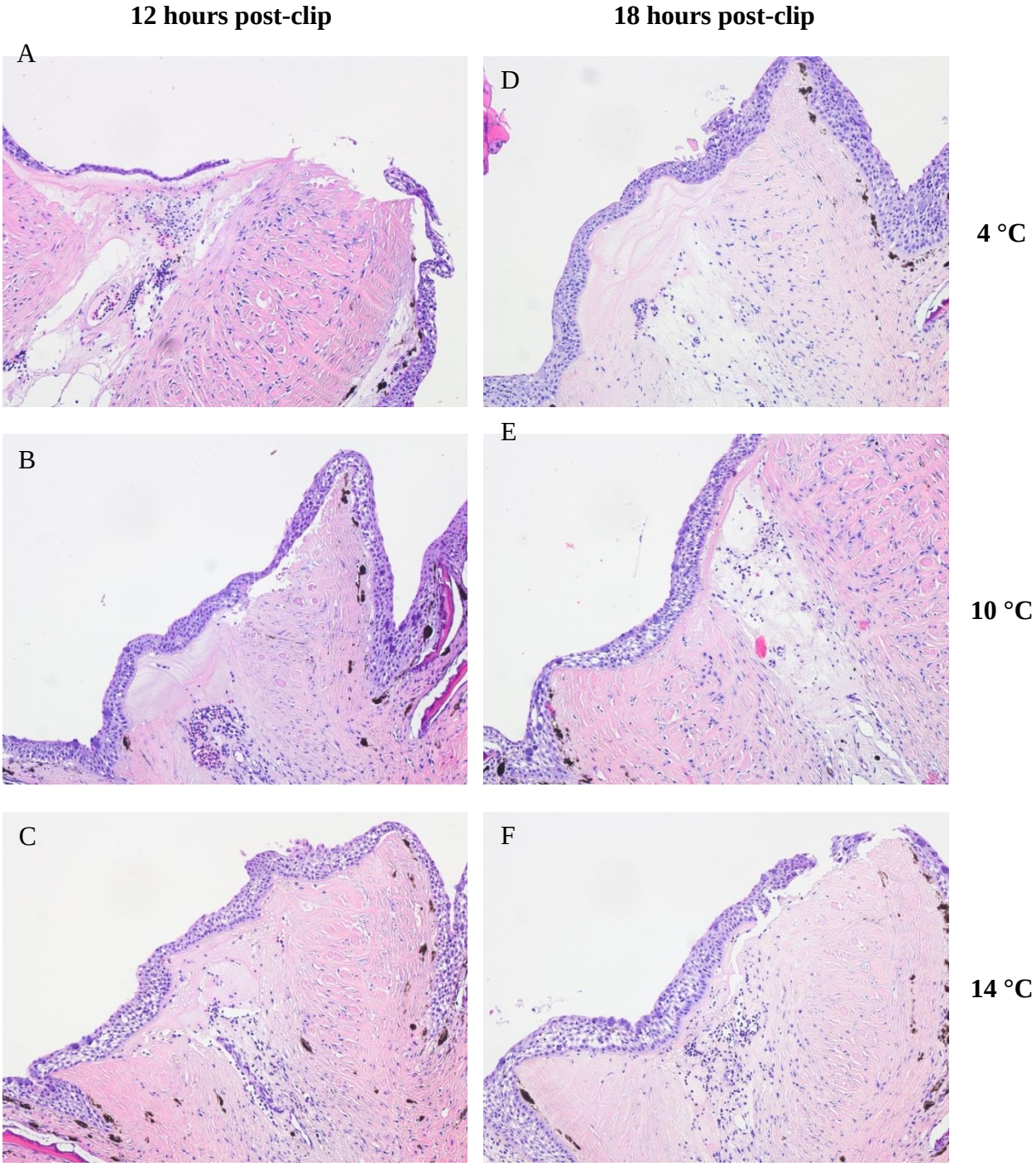


Figure 10. Illustrating wound closure and initial healing following adipose fin clipping; (A) 24h post-clip, 4 °C; (B) 24h post-clip, 10 °C; (C) 24h post-clip, 14 °C. (D) 30h post-clip, 4 °C; (E) 30h post-clip, 10 °C; (F) 30h post-clip, 14 °C.

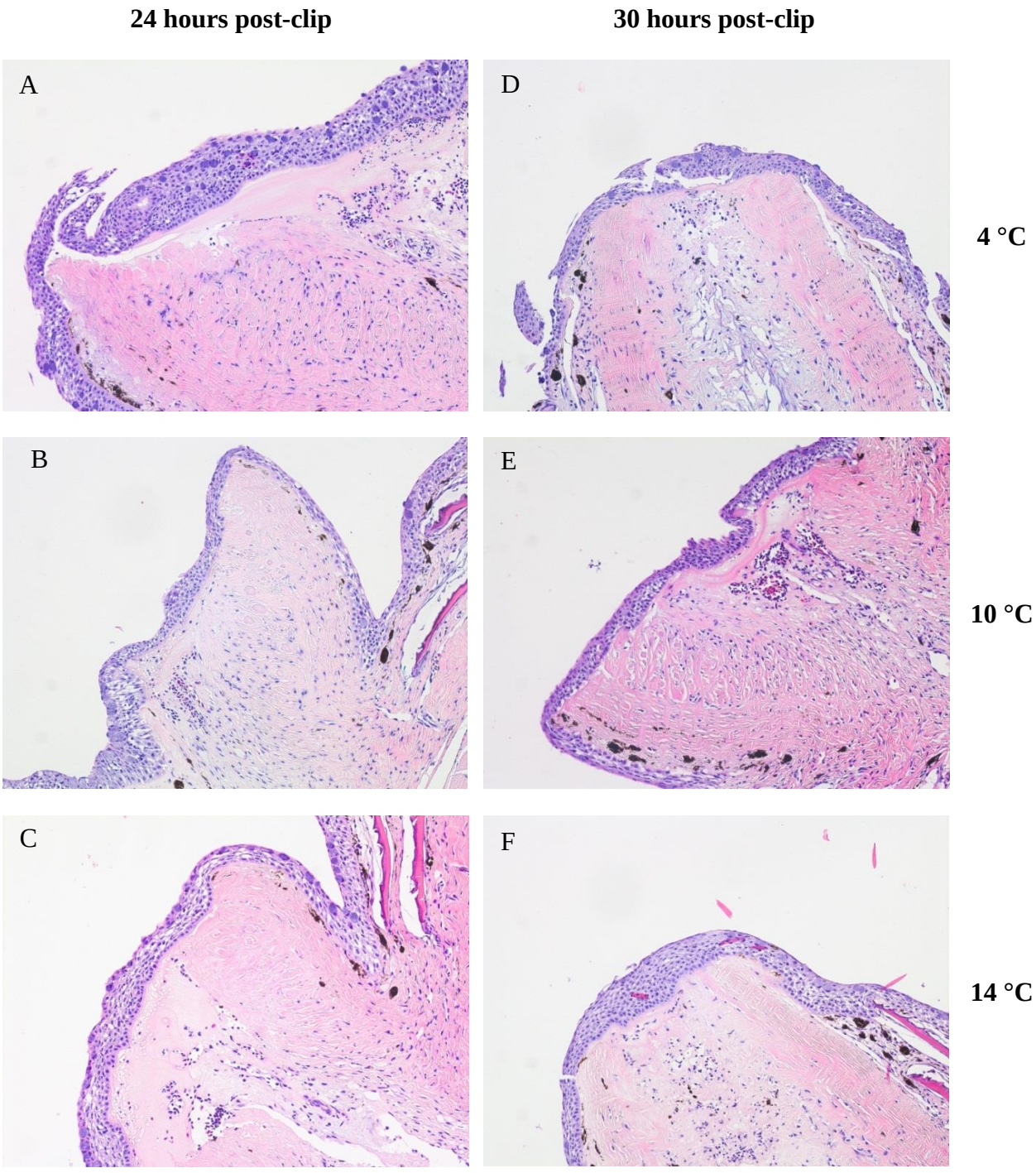


Figure 11. Illustrating wound closure and initial healing following adipose fin clipping; (A) 36h post-clip, 4 °C; (B) 36h post-clip, 10 °C; (C) 36h post-clip, 14 °C. (D) 48h post-clip, 4 °C; (E) 48h post-clip, 10 °C; (F) 48h post-clip, 14 °C.

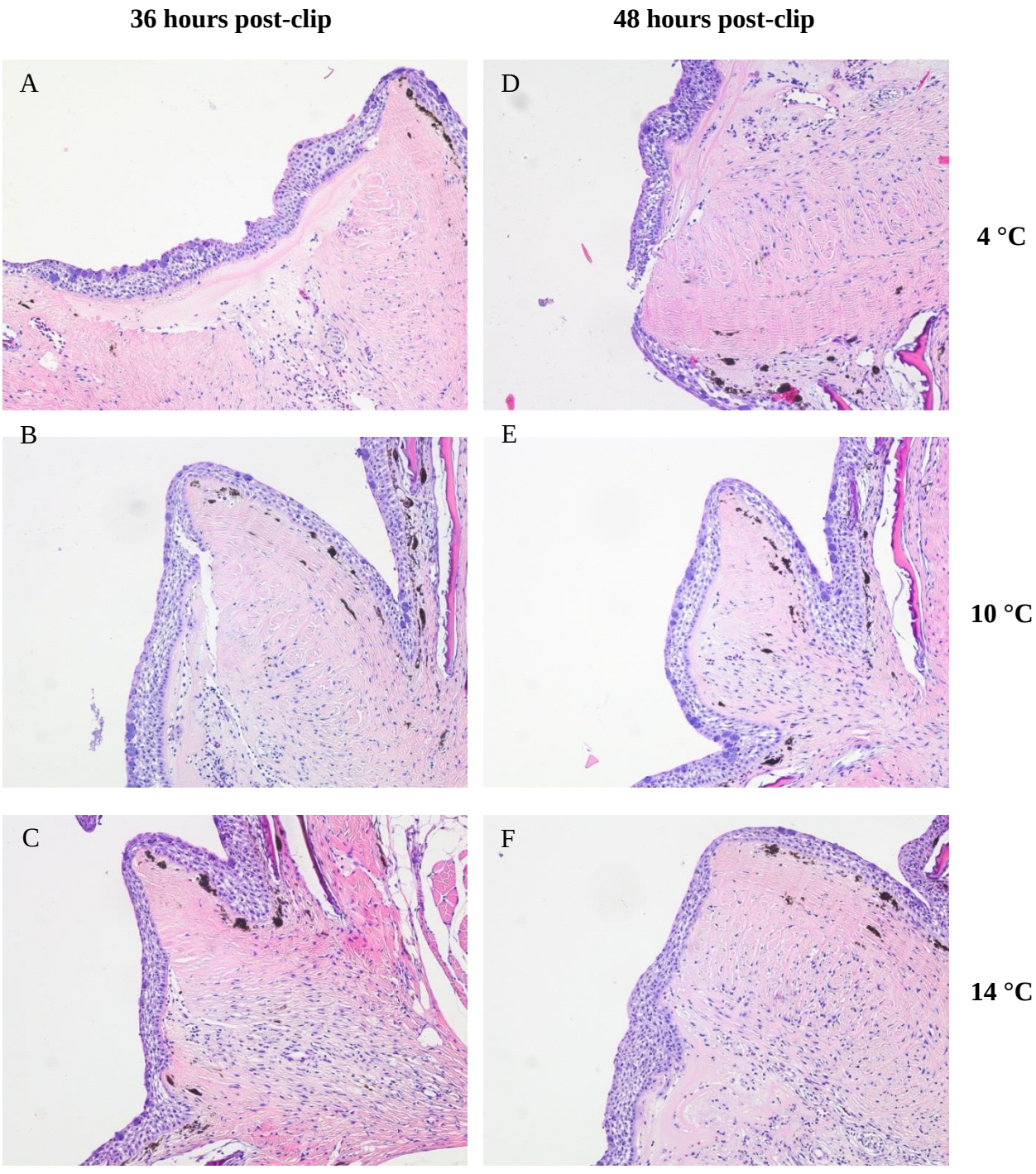
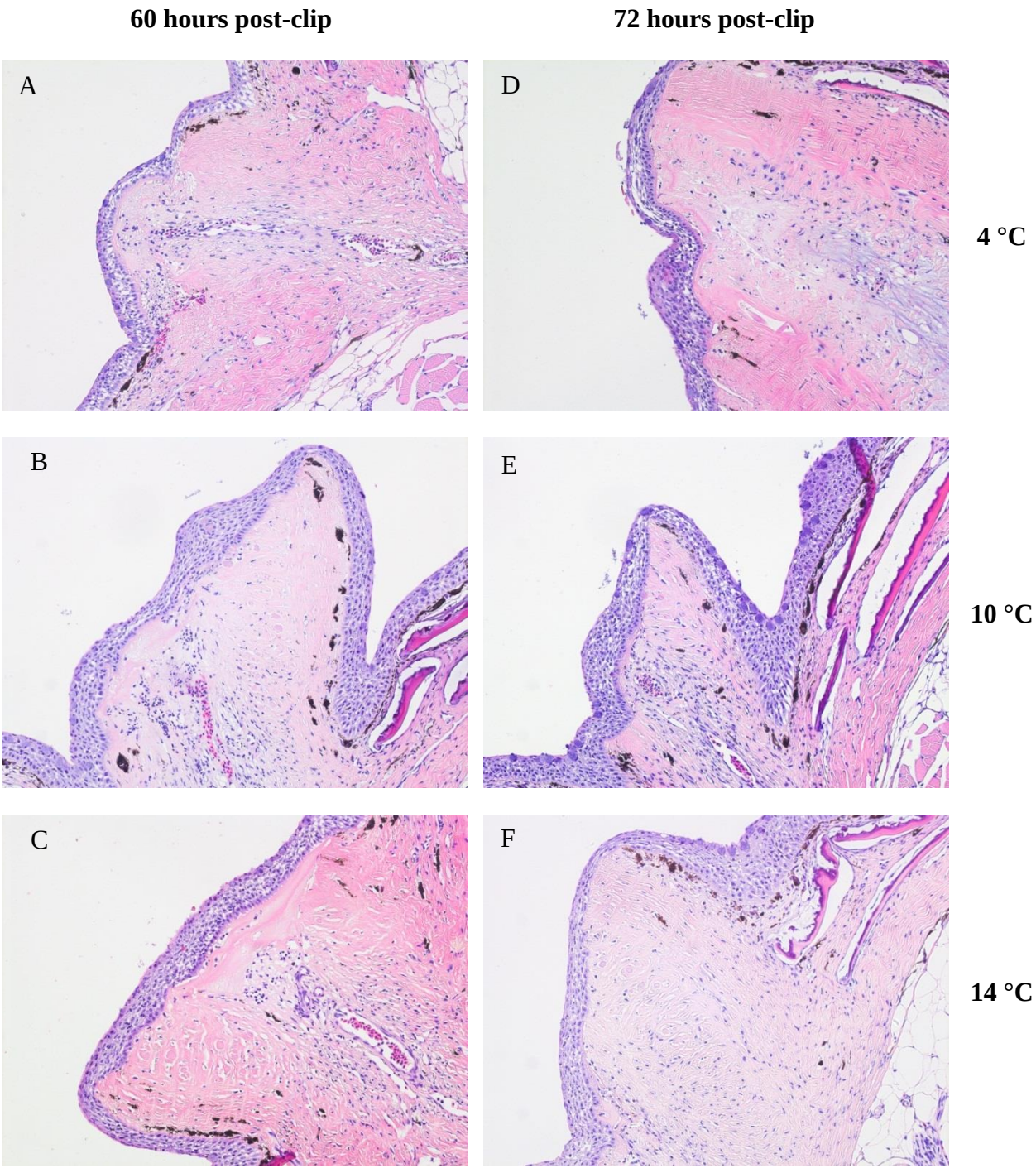


Figure 12. Illustrating wound closure and initial healing following adipose fin clipping; (A) 60h post-clip, 4 °C; (B) 60h post-clip, 10 °C; (C) 60h post-clip, 14 °C. (D) 72h post-clip, 4 °C; (E) 72h post-clip, 10 °C; (F) 72h post-clip, 14 °C.



Summary of WP2: Epidermal healing processes following physical marking of Atlantic salmon pre-smolts

During the current study we wanted to use fish which, if in a commercial setting, would be vaccinated at the same time as being adipose fin clipped. The actual clipping process went very smoothly with no additional stress responses observed other than those expected when conducting routine husbandry procedures. These included an initial increase in swimming velocity which quickly subsided and the fish maintained normal swimming and schooling behaviour (M. Andrews, pers. observ.). Clipping was conducted by a single, highly experienced technician ensuring low variability between the experimental fish. To ensure that water temperatures and water quality remained constant the experiment was conducted in a highly controlled environment, allowing us to describe the actual effect that water temperature may have on the healing rate.

The primary finding from this experiment was that the wound area was covered by a thin epidermal layer 4h post clipping in both the 10 °C and 14 °C groups. The wound area was covered in the lower temperature group of 4 °C at 12h post-clipping. Though it was slower, having a wound close after 12h is far quicker than we had previously estimated and indicates that the fish would experience a very short, if any, period of osmotic stress. The wounds were initially covered by a thin epithelial layer comprising stretched and compressed cells of an undefined nature. Over time the epidermal layer became thicker with the basal cell layer, round cell and superficial cell layers becoming distinguishable, however the cells were not uniform in appearance. Mucous cells also were present relatively soon following clipping, but they took most of the 72h experiment to become well distributed through the epidermal layer. The pigment cells were the only ones that did not return to the wound area, as non-pigmented 'scar' areas are known to persist for some time we may surmise that these cells will take many months to return to the re-epithelized region.

In conclusion, the rate of wound closure following adipose fin clipping was temperature dependent with reduced closure rates occurring at lower water temperatures. Overall wound healing should proceed uninhibited if good husbandry practices are followed; this would reduce the amount of experienced stress.

WP3: Evidence for functional impairment caused by physical marking, and animal welfare assessment of adipose fin clipping

Materials and Methods

This work package was literature based, with the results obtained from the experimental work packages of this project being compared to published manuscripts dealing with related studies as well as the various EU and Norwegian experimental animal regulations. A literature search was initially conducted using ISI Web of Knowledge to determine what information was available for the Atlantic salmon adipose fin as well as external wound healing of Atlantic salmon. Various forms of the following search terms were used:

- Salmon, Wound, Skin, Healing, Fin, Adipose

This produced 6 publications, three describing behavioural or developmental aspects of the adipose fin (Kadri *et al.*, 1997; Næsje *et al.*, 1988; Westley *et al.*, 2008) and three describing morphological aspects (Buckland-Nicks *et al.*, 2012; Harris & Hunt, 1975; Pittman *et al.*, 2011). Due to the paucity of these results we decided to expand the search to include skin/fin healing of any fish species. Adding the following new search terms to those mentioned above we conducted further literature searches:

- Fish, Culture, Damage

After running numerous searches using various search term combinations we compiled a diverse library which we cleaned up by removing completely unrelated references creating a final library consisting of 52 references.

We then decided to search for publications dealing with marking and tagging of experimental and wild fish. We ran numerous searches on ISI Web of Knowledge using variations of the following search terms:

Marking, Tagging, Fish, Wild, Culture, Clipping, Fin, Adipose, Branding, Implant

As with the previous search we cleaned up the results by removing unrelated references ending up with 48 references. A third and final batch of searches were conducted on ISI Web of

Knowledge to collect references dealing with fish welfare, we used variations of the following search terms:

- Fish, Animal, Experiment, Culture, Welfare, Ethics, Laws

Due to the general nature of this aspect of the study we retained many outlying manuscripts and compiled a library of 105 references.

These reference libraries were then read to determine whether any had dealt with the primary question posed by this project, i.e. what may be the welfare impact of adipose fin clipping on Atlantic salmon. We then compiled the following discussion to further explain how our experimental results may aid in determining the welfare impact of adipose fin clipping on Atlantic salmon. In addition we referred to the EU convention for the protection of vertebrate animals used for experimental and other scientific purposes, the Norwegian regulation of animal experimentation, and general animal welfare laws.

Discussion; experimental evidence and literature review

Over the past decade the need for improved understanding of cultured and experimental fish welfare status has been steadily increasing. However, agreement on the ability of fish to experience pain and/or suffering is on-going due to incomplete knowledge of these responses in fish as compared to those of terrestrial animals (Braithwaite and Huntingford, 2004; Huntingford *et al.*, 2006). General handling and husbandry practices have been improved, thus improving overall fish welfare, however further work is needed as it is not clear what effect some commonly employed techniques may have on the fish (Ashley, 2007; Braithwaite and Salvanes, 2010).

For many years there has been interest in the welfare of vertebrates used for experimental purposes which lead to the formation of the EU animal protection laws which were updated in 2005 (*see* EU Convention website). This primarily deals with terrestrial animals with only a short section dealing with fish. The primary recommendation is that experimental fish should be kept in similar conditions as what they would in commercial settings, be supplied regularly with the appropriate diet and be subjected to as little stress as possible. In addition they recommend

that the least invasive tagging method should be used, by invasive they imply that the fish should not experience prolonged stress or pain following marking. In order to further improve the overall welfare of experimental animals, Norway compiled a set of requirements and regulations building on those provided by the EU (*see* NRC webpage). These regulations ensured that all experiments using animals must first go through a thorough approval process conducted by the Norwegian Animal Research Authority (NARA). In these regulations it was stated that there is still insufficient knowledge of the impact that the necessary general handling procedures (such as marking of batches of fish) may have on the overall fish welfare. However it implies that efforts should be made to ensure that the least invasive procedures are used and that improved knowledge is needed to fully understand the impact that commonly employed handling practices may have on cultured and experimental fish as well as other vertebrates. This was the basis for conducting the current project to identify the welfare impact of adipose fin clipping on Atlantic salmon.

Adipose fin clipping is a commonly employed marking technique used by both industry and researchers for group identification of salmonids. It is considered a useful marking technique as it is long lasting and is visible without the need for additional identification equipment (Vander Haegen *et al.* 2005). The difficulty with identifying the impact of adipose fin clipping, or any marking technique, is that there are numerous other stressful handling activities that occur concurrently. Some of the common husbandry techniques used during many procedures are to reduce the water in the tank or confining the fish in a smaller area of the sea cage, scooping them up with nets, undergoing anaesthesia, and then recovery from anaesthesia (Sharpe *et al.*, 1998). These procedures are characterised by the EU Convention and the NRC as basic needs for ensuring good living standards for both experiment and cultured fish (*see* EU Convention and NRC websites). On top of all of these routine procedures additional procedures such as marking, vaccination, and weight/health checks are conducted relatively frequently. An inability to isolate these activities makes it very difficult to determine how stressful a single activity may be, therefore reducing stress and the associated detrimental effects is very important to ensure improved growth, production and welfare (Ashley, 2007).

A drawback to using adipose fin clipping, or indeed any other physical marking technique, would be the additional time and monetary costs that are incurred. It has been suggested that a

marking apparatus may be added to the automated vaccine machines, this would reduce the need for additional participants and increases processing speed. However this is not an option for smaller farms which often do not have access to such automated vaccine technology. Thus, adipose fin clipping is not a cost free procedure, but will increase the time and manpower needed at the time of vaccination, albeit remaining a low-cost method when compared to other physical marking techniques.

A primary aim during this project (particularly in WP 2.3) was to reduce any external factors may negatively affect the healing rates, thus providing accurate results. The fish were acclimated for a week prior to commencing the experiment, in addition they were transferred and handled carefully and swiftly in order to return the fish to their holding tanks as soon as possible. During the experiment we observed the fish to determine whether they were exhibiting abnormal behaviour. We found that they did not exhibit any obvious adverse side effects to the handling as they quickly returned to normal swimming behaviour following handling and marking. We can surmise that our efforts prevented additional stress allowing the healing processes following adipose fin clipping to proceed unhindered.

Observations made during past unpublished experiments indicated that the wound was closed three days post adipose fin clipping (P. Midtlyng, unpublished observation). The results from this project illustrated a much faster wound closure rate than expected, with the closure rate being highly water temperature dependent. Lower water temperatures of 4 °C resulted in the wound taking up to 12h to be covered by a thin epidermal cell layer; this was markedly slower than those held at 10 °C and 14 °C which took 4h to close. Though the fish at lower temperatures did experience slower wound closure, it was still faster than originally expected. This rapid wound closure rate suggests that this method does indeed fall under what the European Convention deems as minimally invasive (*see* EU Convention website). It has been determined that 4°C is close to the minimum acceptable water temperature to be used in aquaculture, this is primarily due to the fact that the fish would not fare well if removed from the water when the air temperatures are too low. However facilities in the more northern areas of Norway, which have longer periods of cold water may have no choice but to operate at these temperatures. These fish may then experience slower wound closure rates as compared to those in the warmer southern regions. Although we did not observe signs of wound infection in the current study, care should

be taken to ensure that high bacterial loads in the water should be avoided during and immediately post clipping in order to minimize the possibility of secondary infections. However we understand that maintaining optimal water quality levels is challenging, particularly in recirculation facilities. We nevertheless suggest that the microbial quality of the water in anaesthesia bath and wakeup tank is being monitored and/or managed, because bacteria and fungi tend to prove the primary culprits in any secondary infection development.

Another factor that may impact adipose fin clipped fish are side effects resulting from skin damage, including altered osmotic balance and the introduction of infectious agents which would reduce overall fish welfare. Skin damage is linked to the onset of osmo-regulatory problems and is often recorded as a side effect in parasitized fish (Dawson *et al.*, 1998; Pettersen *et al.*, 2013). However, fish parasitized by either *Gyrodactylus salmonis* or *Lepeophtheirus salmonis* experience high levels of stress which has been linked to reduced skin healing ability. In addition these parasites are often present in groups causing a number of skin lesions varying in size (Pettersen *et al.*, 2013), and despite receiving higher numbers of wounds many parasitized fish recover fully. This suggests that healthy fish being adipose fin clipped receiving, in the case of the current experiment, a wound with an approximate area of 0,16 cm^2 would experience very slight impact, if any, on their welfare state.

The final aspect we will discuss is evaluating the functional impact of adipose fin clipping on the Atlantic salmon; this is considered problematic as the biological function of the adipose fin is not fully understood. Though the function of the adipose fin remains under discussion it has been found to be sexually dimorphic in the Atlantic salmon, with males having larger adipose fins than females (Haugland *et al.*, 2011; Næsje *et al.*, 1988). These authors found that male Atlantic salmon experienced an increase in size of the adipose fin as the breeding season approached, thus supporting the theory that it functions as a secondary sexual characteristic. The adipose fin has been hypothesised as having a hydrodynamic role by acting as a passive pre-caudal sensor of turbulent flow (Reimchen and Temple, 2004). The description of the presence of a neural network in the adipose fin by Buckland-Nicks *et al.* (2012) supports the proposed hydrodynamic role, in addition the authors stated that this may mean that fin clipping would affect the swimming ability. However, Reimchen and Temple (2004) found no significant changes to the swimming activity following adipose fin clipping, this disparity emphasises that the adipose fin

may be a rudimentary appendix that may not have a true functionality in the farming environment.

Vander Haegen *et al.* (2005) undertook a study to determine whether fin clipping does indeed affect growth and survival in the wild. They used Chinook salmon *Oncorhynchus tshawytscha* (Walbaum, 1792) and found that adipose fin clipping had no effect on juvenile to adult survival. In addition they found that adipose fin clipped juveniles did not stray when they returned to their release rivers. It was concluded that adipose fin clipping was not detrimental to the health of the fish and is an acceptable method for batch marking.

In summary, removal of the adipose fin should not result in increased mortality or decreased overall welfare if it is conducted in a professional manner. This entails ensuring that the overall environmental and handling conditions are well controlled, thus not allowing for any secondary infections or other negative effects to occur. Wound closure rates were dependent on water temperatures; however, even at low water temperatures it occurred fairly rapidly and by doing so reduced the likelihood of infections from setting in. And finally, removal of the adipose fin did not cause a reduction in survival or growth and has no adverse effect on the fish's ability to return to their river of origin. The fact that the wounds closed rapidly, no secondary infections occurred, and few behavioural changes were observed; lend us to conclude that adipose fin clipping may be classified as minimally invasive method. Thus it would also constitute a "humane" method as defined in the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes, well suitable for batch marking large numbers of farmed Atlantic salmon,

References

Ashley, P.J. (2007). Fish welfare: Current issues in aquaculture. *Applied Animal Behaviour Science*, 104, 199-235.

Braithwaite, V.A. & Huntingford, F.A. (2004). Fish welfare: do fish have the capacity for pain perception and suffering? *Animal Welfare*, 13, S87-92.

Braithwaite, V.A. & Salvanes, A.G.V. (2010). Aquaculture and restocking: implications for conservation and welfare. *Animal Welfare*, 19, 139-149.

Buckland-Nicks, J.A., Gillis, M. & Reimchen, T.E. (2012). Neural network detected in a presumed vestigial trait: ultrastructure of the salmonid adipose fin. *Proceedings of the Royal Society B*, 279, 553-563.

Chart, T.E. & Bergersen, E.P. (1988). Methods for long-term identification of salmonids: a review. *Biological Report*, 88 (37), 1-18.

Dawson, L.H.J., Pike, A.W., Houlihan, D.F. & McVicar, A.H. (1998). Effects of salmon lice *Lepeophtheirus salmonis* on sea trout *Salmo trutta* at different times after seawater transfer. *Diseases of Aquatic Organisms*, 33, 179-186.

Dietrich, J.P. & Cunjak, R.A. (2006). Evaluation of the impacts of carlin tags, fin clips, and panjet tattoos on juvenile Atlantic salmon. *North American Journal of Fisheries Management*, 26, 163-169.

Evrard, G. (2005). Use and effect of freeze branding on roach (*Rutilus rutilus* L.). *Bulletin Francais de la Pêche et de la Pisciculture*, 374, 35-42.

FHL webpage. Styrket miljøfokus i havbruksnæringen"; http://www.fhl.no/miljoe/dette-er-miljoetiltakene-article3846-24.html

FitzGerald, J.L., Sheehan, T.F. & Kocik, J.F. (2012). Visibility of visual implant elastomer tags in Atlantic salmon reared for two years in marine net-pens. *North American Journal of Fisheries Management*, 24, 222-227.

Friedland, K.D., Esteves, C., Hansen, L.P. and Lund, R.A. (1994). Discrimination of Norwegian farmed, ranchend and wild-origin Atlantic salmon, Salmo salar L., by image processing. *Fisheries Management and Ecology*, 1, 117-128.

Gunnes, K. and Refstie, T. (1980). Cold branding and fin clipping for marking of salmonids. *Aquaculture*, 19, 295-299.

Harris, J.E. and Hunt, S. (1975). The fine structure of the epidermis of two species of salmonid fish, the Atlantic salmon (*Salmo salar* L.) and the brown trout (*Salmo trutta* L.). I. General organisation and filament-containing cells. *Cell and Tissue Research*, 157, 553-565.

Haugland, T., Rudolfsen, G., Figenschou, L. & Folstad, I. (2011). Is the adipose fin and lower jaw (kype) related to social dominance in male Arctic charr *Salvelinus alpinus*? *Journal of Fish Biology*, 79, 1076-1083.

Huntingford, F.A., Adams, C., Braithewaite, V.A., Kadri, S., Pottinger, T.G., Sandøe, P. and Turnbull, J.F. (2006). Current issues in fish welfare. *Journal of Fish Biology*, 68, 332-372.

Kadri, S., Metcalfe, N.B., Huntingford, F.A., Thorpe, J.E. and Mitchell, D.F. (1997). Early morphological predictors of maturity in one-sea-winter Atlantic salmon. *Aquaculture International*, 5, 41-50.

Kleingeld, D.W. (2005). Aspects of animal welfare with regard to the production of farmed fish in aquaculture systems. *Deutsche Tierärztliche Wochenschrift*, 112, 100-103 [in German].

Lund, R.A., Hansen, L.P. and Järvi, T. (1989). Identifikasjon av rømt oppdrettslaks ved ytre morfologi, finnestørrelse og skjellkarakter. NINA fagrapport 001.54.

Lund, R.A., Midtlyng, P.J. and Hansen, L.P. (1997). Post-vaccination intra-abdominal adhesions as a marker to identify Atlantic salmon, *Salmo salar* L., escaped from commercial fish farms. *Aquaculture*, 154, 27-37.

Needham, T. (1988). Sea water cage culture of salmonids. In: *Salmon and trout farming*, Eds. Laird, L.M. and Needham, T. pp 271.

Nielsen, L.A. (1992). Methods of marking fish and shellfish. American Fisheries Society Special Publication no 23, 208 pp.

Næsje, T.F., Hansen, L.P. and Järvi, T. (1988). Sexual dimorphism in the adipose fin of Atlantic salmon, *Salmo salar* L. *Journal of Fish Biology***,** 33, 955-956.

Pettersen, R.A., Hytterød, S., Vøllestad, L.A. & Mo, T.A. (2013). Osmoregulatorydisturbances in Atlantic salmon, *Salmo salar* L., caused by the monogenean *Gyrodactylus salaris*. *Journal of Fish Diseases*, 36, 67-70.

Pittman, K., Sourd, P., Ravnøy, B., Espeland, Ø., Fiksdal, I.U., Oen, T., Pittman, A., Redmond, K. and Sweetman, J. (2011). Novel method for quantifying salmonid mucous cells. *Journal of Fish Diseases*, 34, 931-936.

Reimchen, T.E. & Temple, N.F. (2004). Hydrodynamic and phylogenetic aspects of the adipose fin in fishes. *Canadian Journal of Zoology*, 82, 910-916.

Sharpe, C.S., Thompson, D.A., Blankenship, H.L. & Schreck, C.B. (1998). Effects of routine handling and tagging procedures on physiological stress responses in juvenile Chinook salmon. *Progressive Fish Culturist*, 60, 81-87.

Vander Haegen, G.E., Blankenship, H.L., Hoffmann, A. & Thompson, D.A. (2005). The effects of adipose fin clipping and coded wire tagging on the survival and growth of spring chinook salmon. *North American Journal of Fisheries Management*, 25, 1161-1170.

Wahli, T., Verlhaq, V., Girling, P., Gabaudan, J. and Aebischer, C. (2003). Influence of dietary vitamin C on the wound healing process in rainbow trout. *Aquaculture*, 225, 371-386.

Westley, P.A.H., Carlson, S.M. and Quinn, T.P. (2008). Among-population variation in adipose fin size parallels the expression of other secondary sexual characteristics in sockeye salmon (*Oncorhynchus nerka*). *Environmental Biology of Fishes*, 81, 439-446.

Wydowski, R. and Emery, L. (1983). Tagging and marking. Pp 215-237 in L.A. Nielsen and D.L. Johnson, eds. Fisheries techniques. American Fisheries Society, Bethesda, Maryland.

Webpages:

EU convention for the protection of vertebrate animals used for experimental and other scientific purposes. http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm and http://www.coe.int/t/e/legal_affairs/legal_co-operation/biological_safety_and_use_of_animals/laboratory_animals/2006/Cons123(2006)3AppendixA_en.pdf [cited 22 March 2013]

FAO (2013). Aquaculture topics and activities. Aquaculture. In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 30 November 2012. [Cited 24 January 2013].

Forskningsradet. http://www.forskningsradet.no [Cited 22 March 2013]