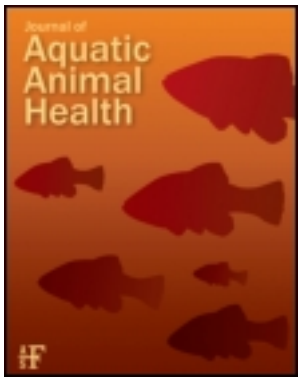
On: 25 May 2012, At: 04:04
Publisher: Taylor & Francis
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK




Journal of Aquatic Animal Health

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/uahh20

# Prevention of Ulcer Disease in Goldfish by Means of Vaccination

Peter A. W. Robertson [a] , Dawn A. Austin [a] & Brian Austin [a]

[a] School of Life Sciences, John Muir Building, Heriot-Watt University, Riccarton, Edinburgh, EH14 4AS, Scotland, UK

Available online: 09 Jan 2011

To cite this article: Peter A. W. Robertson, Dawn A. Austin & Brian Austin (2005): Prevention of Ulcer Disease in Goldfish by Means of Vaccination, Journal of Aquatic Animal Health, 17:2, 203-209

To link to this article: http://dx.doi.org/10.1577/H04-038.1

*Journal of Aquatic Animal Health* 17:203–209, 2005

DOI: 10.1577/H04-038.1

[Article]

# Prevention of Ulcer Disease in Goldfish by Means of Vaccination

PETER A. W. ROBERTSON, DAWN A. AUSTIN, AND BRIAN AUSTIN*

*School of Life Sciences, John Muir Building, Heriot-Watt University, Riccarton, Edinburgh EH14 4AS, Scotland, UK*

*Abstract.*—A vaccine comprising cells of *Aeromonas bestiarum* grown in tryptic soy broth and atypical *A. salmonicida* cells produced in iron-limited and iron-supplemented media protected goldfish *Carassius auratus* when administered by immersion (dosage $\approx 5 \times 10^7$ cells/mL for 60 s) followed after 28 d by an oral booster (dosage $= 5 \times 10^7$ cells/g of feed), which was fed for 7 d so that each fish received about 1 g of vaccine-containing feed. After challenge by intramuscular injection of a virulent culture of atypical *A. salmonicida*, the relative percent survival (RPS) was more than 90%. The approach was more successful than using a commercial furunculosis vaccine with or without supplementation with *A. bestiarum* or atypical *A. salmonicida* cells. Moreover, a smooth derivative of the virulent rough culture of atypical *A. salmonicida* was less effective as a vaccine candidate, yielding an RPS of only 65%. Low antibody titers of 1:39–1:396 were found in the vaccinated fish. The vaccinated fish had a significantly higher proportion of dead head kidney macrophages (10.9 ± 3.5%; $P = 0.0149$) than did the controls (6.8 ± 3.1%). However, differences in the number of erythrocytes and leukocytes, the level of phagocytic and lysozyme activities, and the proportion of lymphocytes, monocytes, and polymorphonuclear cells were not statistically significant between the two groups.



The origin of vaccine development for furunculosis may be traced to the work of Duff (1942), who used an orally administered chloroform-inactivated suspension of whole cells and successfully protected cutthroat trout *Oncorhynchus clarkii*. Subsequent work rarely equaled or surpassed this original study (see Austin and Austin 1999). Goldfish *Carassius auratus* may become infected with so-called atypical *Aeromonas salmonicida*, which leads to the development of unsightly ulcers (Austin and Austin 1999). Often, the ulcers are populated with mixed cultures in which motile aeromonads, typically identified as *A. hydrophila*, are common (Austin and Austin 1999). There is a lack of evidence of synergism between bacterial groups leading to exacerbated disease, and these motile aeromonads may be secondary invaders of ulcerated tissue (Austin and Austin 1999). Previous work to develop vaccines for the control of ulcer disease of cyprinids has met with mixed success (e.g., Kozinska and Guz 2004), often because of the lack of a reproducible challenge method (Austin and Austin 1999). With the current success in salmonids of formalin-inactivated *A. salmonicida* preparations (e.g., Durbin et al. 1999), we considered it timely to reexamine the potential of vaccines for controlling ulcer disease in ornamental fish.

## Methods

*Source of bacterial cultures.*—One hundred eighty-three live, ulcerated ornamental fish—goldfish and koi carp *Cyprinus carpio*—were received from wholesale and retail outlets in England. The fish were killed by overdose with anesthetic (MS-222; Sigma, Basingstoke, UK) and, using flame-sterilized scalpels, we cut into the muscle leading away from the ulcers. Loopfuls of material were spread over the surface of 5% (volume basis [v/v]) horse blood in nutrient agar, 5% (v/v) sheep blood in nutrient agar, and tryptic soy agar (TSA; all materials from Oxoid, Basingstoke, UK) and incubated room temperature (18–20°C) for 7 d. Plates with dense growth representing no more than two dominant colony types were examined further, and pure cultures were obtained by streaking and restreaking on fresh media. Thus, 42 pure cultures were obtained, which we equated with *A. bestiarum* (7 cultures) and *A. salmonicida* (35 cultures) after comparing with published characteristics (Ali et al. 1996; Austin et al. 1998). In subsequent vaccination experiments, we used three cultures from goldfish, ORN2 (= *A. bestiarum*) and ORN6 and ORN6a (= brown pigmented, autoagglutinating, rough [A-layer$^+$] atypical *A. salmonicida*), which had been determined to be virulent in separate infectivity experiments. Stock cultures

* Corresponding author: b.austin@hw.ac.uk

Received June 23, 2004; accepted September 12, 2004
Published online June 2, 2005

TABLE 1.—Vaccine formulations evaluated in a study of ulcer disease in goldfish. The abbreviations ORN2 and ORN6 refer to cultures of *Acromonas bestiarum* and *A. salmonicida*; TSB refers to tryptic soy broth.

| Vaccine | Immersion | Oral | Oral boost |
|---|---|---|---|
| Furovac 5 | + | | + |
| Furovac 5 supplemented with iron-limited preparations of ORN2 and ORN6 | + | | + |
| Furovac 5 supplemented with iron-limited and iron-supplemented preparations of ORN2 and ORN6 | + | | + |
| Iron-restricted preparation of ORN6 | + | | |
| Iron-supplemented preparation of ORN6 | + | | |
| Iron-limited preparation of ORN6 and cells of ORN2 grown in TSB | + | | + |
| Iron-supplemented preparation of ORN6 and cells of ORN2 grown in TSB | + | | + |
| Iron-limited and iron-supplemented preparation of ORN2 and ORN6 | + | | + |
| Iron-limited and iron-supplemented preparation of ORN6 and cells of ORN2 grown in TSB | + | + | + |
| Cells of ORN2 and ORN6 grown in TSB | + | | + |
| Iron-limited and iron-supplemented preparation of a smooth culture of ORN6 and cells of ORN2 grown in TSB | + | | + |



were stored at −70°C either in tryptic soy broth (TSB; Oxoid) containing 15% (v/v) glycerol (Sigma) or as freeze-dried preparations. Routinely, the cultures were maintained on TSA plates and slopes at room temperature with subculturing every 7–14 d.

*Fish stocks.*—Stocks of goldfish approximately 6 cm long were obtained from commercial sites in England and were maintained in aerated, recirculating freshwater at 17°C. We examined their health status (Austin and Austin 1989) and used only stocks deemed to be healthy and, in particular, free of ulceration in subsequent experiments.

*Vaccines.*—A commercial vaccine against *A. salmonicida* (Furovac 5; Schering Plough Aquaculture Division, Saffron Walden, UK) was used with and without supplementation with formalin-inactivated (to 0.2% [v/v] with incubation at room temperature for 7 d) cells of *A. bestiarum* ORN2, atypical *A. salmonicida* ORN6, or both. Additional preparations were based exclusively on the laboratory-produced inactivated cell suspensions, and the formulated products contained equal numbers of cells of each component (Table 1). The cultures were grown for 48 h at room temperature in TSB with or without 0.1 mM dipyridyl (Sigma) to induce iron-deficient conditions and the production of iron-regulated outer membrane proteins (IROMPs) (Durbin et al. 1999) or with 0.1 mM ferric chloride (Sigma) for iron supplementation. Additionally, ORN6 was repeatedly subcultured every 7 d with incubation at 30°C to induce the development of nonautoagglutinating smooth cultures (Austin and Austin 1999), which we used to produce vaccines. After inactivation with formalin, the cultures were checked for inactivation by inoculating 1.0-mL volumes into 9.0 mL of TSB and incubating at room temperature for 7 d. Then, 0.1-mL volumes of this incubated material were spread over the surface of triplicate plates of TSA and further incubated for up to 7 d at room temperature. The absence of growth was used as an indication of inactivation. The formalin-treated cultures were stored in the original inactivated growth medium at 4°C until required.

*Vaccination regimes.*—Groups of 100 goldfish were vaccinated by immersion or orally, and 28 d after the initial procedure were treated with or without immersion or oral booster doses. For immersion vaccination, we diluted the stock vaccine in tank water to about $5 \times 10^7$ cells/mL, as determined by reading a sample on a hemocytometer slide (Improved Neubauer type; Merck, Lutterworth, UK) at a magnification of 400× on an Axiophot microscope (Carl Zeiss, Welwyn Garden City, UK**).** The fish were immersed in this diluted vaccine for 60 s before being returned to the holding tanks. For oral vaccination, we mixed the vaccine with an equal volume of oralizer (Schering Plough Aquaculture; International Patent WO 92/06599) that contained fish oil and emulsifying agent. We emulsified the mixture at high-shear forces for 20 min in a Silverson (Waterside, Chesham, UK) homogenizer, and then mixed this emulsion with commercial pelleted feed to achieve a dose equivalent to $5 \times 10^7$ cells/g of feed. This preparation was fed on demand to the fish, three times daily for 7 d, such that each fish received about 1.0 g of feed in total. Additional feeding on demand was with the same commercial pelleted feed, minus the added emulsion. Each vaccination procedure was carried out once for each set of experiments, but over the course of the work, the vaccines were evaluated four times.

*Challenge experiments.*—The fish were chal-

lenged 28 d after initial vaccination or booster with atypical *A. salmonicida* ORN6a by intramuscular injection of this organism at $2 \times 10^2$ cells/fish. Preliminary experiments revealed that this was the equivalent of an LD80 dose (the dose at which 80% of infected fish died), with an outcome of ulceration and mortality. Thus, we grew the infectious culture overnight at room temperature in TSB, centrifuged it ($4000 \times g$ at 4°C for 15 min), washed it twice, resuspended it in 0.9% (w/v) saline, and determined the cell count of the culture by viewing on a hemocytometer slide (Improved Neubauer Type) at a magnification of 400× on a Carl Zeiss Axiophot microscope. The fish were examined daily for 21 d, and the number of mortalities recorded and used to calculate the relative percent survival (RPS; Amend 1981). We examined dead and moribund fish microbiologically to confirm the presence of atypical *A. salmonicida* (Austin et al. 1998).

*Statistics*.—Quantitative data were examined by a range of statistical methods, including the calculation of standard deviations, Student's *t*-test, and the Mann–Whitney test (Steele and Torrie 1981), contained on the INSTAT 2.01 for Macintosh software (GraphPad Software, San Diego, California). We used the parametric Student's *t*-test to examine all immunology data.Where the data did not follow Gaussian distribution patterns, we also used the nonparametric Mann–Whitney test to determine statistical significance.

*Immunology*.—The methods used to examine changes in immunological parameters after vaccination have been described elsewhere (Irianto and Austin 2002). In brief, blood was collected from freshly killed fish (10 fish per group) by venipuncture, and then diluted 10-fold and 100-fold in phosphate-buffered saline at pH 7.2 (PBS; Oxoid). We examine the diluted sample microscopically using a hemocytometer slide (Improved Neubauer Type) to determine the number of leukocytes and erythrocytes. We also examined blood films to determine the relative proportion of leukocytes, that is, of polymorphonuclear granulocytes, lymphocytes, and monocytes. The remainder of the blood was transferred to evacuated collection tubes containing clot-activating gel (Vacuettes; Greiner, Gloucester, UK), and allowed to clot by incubation at room temperature for 1 h, and stored overnight at 4°C. The serum obtained was then removed and stored at −70°C until required.

Lysozyme activity in serum samples was determined by the reduction in optical density at 570 nm of a suspension of *Micrococcus luteus* (formerly *M. lysodeikticus*) obtained from the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, UK; 9278) as described by Irianto and Austin (2002). We also analyzed the serum samples with an enzyme-linked immunosorbent assay (ELISA) to determine the presence and titer of antibodies to atypical *A. salmonicida* ORN6 (Durbin et al. 1999).

From the same fish we obtained the kidneys and used them to determine the relative proportion of dead and live macrophages and macrophage phagocytic activity (Irianto and Austin 2002).

## Results

### *Vaccination*

The results revealed that the commercial furunculosis vaccine (Furovac 5) administered by immersion with or without an oral booster did not protect goldfish against mortalities when challenged with atypical *A. salmonicida*; the cumulative mortalities over 21 d corresponding to those of the controls (experiment 1; Table 2). Moreover, supplementing this vaccine with formalin-inactivated cells of ORN2 and ORN6, grown in conditions of iron-limitation, did not improve protection (experiment 2; Table 2). Indeed, for fish immersed in the iron-limited and iron-supplemented preparations, the cumulative mortalities exceeded those of the controls (experiment 2; Table 2). Yet supplementing the commercial vaccine with iron-limited and iron-supplemented preparations improved the benefit of immersion vaccination followed by an oral booster after 28 d (Table 2), the cumulative mortalities being less than those of the controls (experiment 2; Table 2). Consequently, we focused solely on vaccines based exclusively around isolates recovered from ornamental fish. Using formalin-treated cells of atypical *A. salmonicida* ORN6 that had been grown in either iron-limited or iron-supplemented conditions was not successful when administered only by immersion, in comparison with the controls (experiment 3; Table 2). Similarly, a mixture of *A. bestiarum* ORN2 and atypical *A. salmonicida* ORN6 cells prepared in iron-limited and iron-supplemented conditions was not effective when administered to goldfish solely by immersion (experiment 4; RPS = 1%; Table 2). But the administration of an oral booster to goldfish after immersion vaccination substantially improved protection (experiment 4; RPS = 81%; Table 2). Here, the cumulative mortality among the vaccinated fish was only16%, compared with 84% total mortalities in the con-

TABLE 2.—Effect of vaccination on survival of groups of 100 goldfish following challenge with atypical *A. salmonicida* (ORN6a). The abbreviation RPS stands for relative percent survival and was calculated according to Amend (1981). See Table 1 for other abbreviations. A plus sign denotes a positive effect, a minus sign no effect.

| Experiment | Vaccine | | | | | | | Vaccination regime | | Mortalities 21 d after challenge (%) | RPS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Iron limited | | Iron supplemented | | TSB | | | | | |
| | Furovac 5 | ORN2 | ORN6 | ORN2 | ORN6 | ORN2 | ORN6 | Immersion | Oral boost | | |
| 1 | + | − | − | − | − | − | − | + | − | 95 | 3 |
| | + | − | − | − | − | − | − | + | + | 98 | 3 |
| | − | − | − | − | − | − | − | − | − | 98 (control) | |
| 2 | + | + | + | − | − | − | − | + | − | 53 | 0 |
| | + | + | + | − | − | − | − | + | + | 37 | 0 |
| | + | + | + | + | + | − | − | + | − | 52 | 0 |
| | + | + | + | + | + | − | − | + | + | 25 | 26 |
| | − | − | − | − | − | − | − | − | − | 34 (control) | |
| 3 | − | − | + | − | − | − | − | + | − | 94 | 2 |
| | − | − | − | − | + | − | − | + | − | 86 | 9 |
| | − | − | − | − | − | − | − | − | − | 95 (control) | |
| 4 | − | + | + | + | + | − | − | + | − | 85 | 1 |
| | − | + | + | + | + | − | − | + | + | 16 | 81 |
| | − | − | − | − | − | − | − | − | − | 84 (control) | |
| 5 | − | + | + | + | + | − | − | + | − | 37 | 62 |
| | − | − | + | − | + | + | − | + | − | 9 | 91 |
| | − | − | + | − | + | + | − | + | + | 7 | 93 |
| | − | − | − | − | − | − | − | − | − | 98 (control) | |
| 6 | − | − | − | − | − | + | + | + | − | 48 | 51 |
| | − | − | + | − | − | + | + | + | + | 65 | 27 |
| | − | − | + | − | + | + | − | + | − | 69 | 23 |
| | − | − | + | − | + | + | − | + | + | 28 | 69 |
| | − | − | − | − | − | − | − | − | − | 89 (control) | |
| 7 | − | − | + | − | + | + | − | + | + | 4 | 96 |
| | − | − | − | − | − | − | − | − | − | 99 (control) | |
| 8 | − | − | +[a] | − | +[a] | + | − | + | + | 35 | 65 |
| | − | − | + | − | + | + | − | + | + | 7 | 93 |
| | − | − | − | − | − | − | − | − | − | 99 (control) | |

[a] Smooth isolate.

trols after 21 d (experiment 4; Table 2). Replacing the iron-limited or iron-supplemented ORN2 component with cells grown in TSB was generally superior; treatment with this preparation after immersion vaccination and challenge improved the protection of goldfish in that the RPS increased from 62% to 91% (experiment 5; Table 2).

The use of ORN2 and ORN6 grown only in TSB gave only moderate levels of protection when administered by immersion with or without an oral booster. When immersion only was used, an RPS of 51% was recorded (experiment 6; Table 2). In a separate experiment, iron-limited and iron-supplemented cells of ORN6 (used separately and in combination) plus cells of ORN2 grown in TSB were used to vaccinate fish by immersion followed by an oral booster with the homologous oralized product. After challenge, 98 unvaccinated controls died compared with 4 in the group that received the iron-limited and iron-supplemented cells of ORN6 (RPS = 96%), 37 of the group that received iron-limited cells of ORN6 by immersion and an oral booster (RPS = 62%), and 44 of the group that received iron-supplemented cells of ORN6 by immersion and an oral booster (RPS = 55%),

Consistently over four replicate experiments, we found that the use of formalin-inactivated cells of atypical *A. salmonicida* ORN6 grown in iron-limited and iron-supplemented conditions together with *A. bestiarum* grown in TSB when applied by immersion with an oral booster provided excellent protection (Table 2). In one experiment, an RPS of 96% was achieved (experiment 7; Table 2); that is, only 4% of the vaccinated fish died, compared with 99% of the controls. Moreover, the surviving vaccinated fish were completely devoid of ulcers. Parallel results were obtained in replicates, with RPS values ranging from 96% to 99% (experiment 7–8). Application of the vaccine solely by the oral route was less successful in that a primary (oral) vaccination led to 68 mortalities (RPS = 12%), whereas an oral booster was only marginally better, with 58 mortalities (RPS = 25%). In comparison, only 6 mortalities (RPS = 92%) occurred when the vaccine was administered by immersion and an oral booster, but the unvaccinated controls

TABLE 3.—Numbers of erythrocytes and leucocytes and phagocytic and lysozyme activity (± SDs) in goldfish (group size = 50; average length = 6 cm) after administering iron-limited and iron-supplemented preparations of ORN6 and cells of ORN2 grown in TSB by immersion followed by an oral booster; see Table 1 for abbreviations. The fish were examined on the same day as challenge, 28 d after feeding the oral booster. *P*-values are given in parentheses; differences are considered statistically significant when $P < 0.05$.

| Group | Erythrocytes ($\times 10^8$/mL) | Leucocytes ($\times 10^7$/mL) | Dead macrophages (%) | Phagocytic activity | Lysozyme activity (units) |
|---|---|---|---|---|---|
| Vaccinates | 7.4 ± 2.6 (0.9283) | 1.3 ± 1.9 (0.7302) | 10.9 ± 3.5 (0.0149) | 48.9 ± 8.7 (0.2774) | 28.7 ± 24.1 (0.3843) |
| Control | 7.6 ± 4.6 | 0.4 ± 0.2 | 6.8 ± 3.1 | 53.3 ± 8.9 | 15.2 ± 17.0 |

had 77 mortalities. Interestingly, a smooth derivative of ORN6 was less successful than the original rough isolate, yielding RPS values of 65% and 93%, respectively (experiment 8; Table 2). The use of formalin-inactivated cells of atypical *A. salmonicida* ORN6 grown in iron-limited and iron-supplemented conditions, together with *A. bestiarum* grown in TSB applied orally with or without an oral booster, was less successful than when applied by immersion followed by an oral booster (experiment 9; Table 2).

*Immunology*

The following data were obtained by using formalin-inactivated cells of atypical *A. salmonicida* ORN6 grown in iron-limited and iron-supplemented conditions and *A. bestiarum* grown in TSB, when applied by immersion with an oral booster. Thus by ELISA, serum antibodies to atypical *A. salmonicida* were detected by the time of challenge in vaccinated fish. Low antibody titers of 1:19–1:99 were consistently recorded among the unvaccinated control goldfish. Titers of 1:39–1:396 were found in the fish that had been vaccinated by immersion (followed by an oral booster) with iron-limited and iron-supplemented preparations of atypical *A. salmonicida* ORN6, or with cells of *A. bestiarum* ORN2 grown in TSB. In contrast, use of Furovac 5 resulted in antibody titers among the vaccinated fish of 1:4–1:99, that is, similar to those of the unvaccinated controls.

Tables 3 and 4 present data for the numbers of erythrocytes and leukocytes; the proportion of phagocytosing macrophages; the relative proportions of lymphocytes, monocytes, and polymorphonuclear cells; and the lysozyme activity. Generally, immersion vaccination and oral boosting with iron-restricted and iron-supplemented cells of atypical *A. salmonicida* ORN6 and the preparation of *A. bestiarum* ORN2 in TSB, goldfish demonstrated more leukocytes, $(1.3 \pm 1.9) \times 10^7$/mL, than did the controls, $(0.4 \pm 0.2) \times 10^7$/mL (Table 3), although these data were not regarded as statistically significant ($P = 0.7302$, Mann–Whitley test). Similarly, a higher proportion of dead macrophages, which did not appear to be necrotic or apoptotic, were recorded for vaccinated fish (10.9 ± 3.5%) than for the controls (6.8 ± 3.1%), a difference that was statistically significant ($P = 0.0149$, Student's *t*-test). Incidentally, all the kidneys appeared to be in good condition, devoid of any visual signs of damage or disease. The number of erythrocytes was similar between vaccinates and controls (Table 3), with differences that were not statistically significant ($P = 0.9283$, Student's *t*-test). Furthermore, the levels of phagocytic macrophages ($P = 0.2774$), lysozyme activity ($P = 0.3843$), and the proportion of lymphocytes ($P = 0.5841$), monocytes ($P = 0.3154$), and polymorphonuclear granulocytes ($P = 0.7096$) in vaccinated and control fish were not regarded as statistically different (Table 3 and 4).

## Discussion

The data reveal that a vaccination strategy may be used successfully to combat mortalities result-

TABLE 4.—Proportion (%) of lymphocytes, monocytes, and polymorphonuclear granulocytes in goldfish blood (average fish length = 6 cm) after administering iron-limited and iron-supplemented preparations of ORN6 and cells of ORN2 grown in TSB by immersion, followed by an oral booster; see Table 1 for abbreviations. The fish were examined on the same day as challenge, 28 d after feeding the oral booster. *P*-values are given in parentheses; differences are considered statistically significant when $P < 0.05$.

| Group | Lymphocytes | Monocytes | Polymorphonuclear granulocytes |
|---|---|---|---|
| Vaccinates | 59.2 ± 10.1 (0.5841) | 8.4 ± 2.6 (0.3154) | 32.4 ± 8.9 (0.7096) |
| Control | 62.6 ± 15.4 | 7.5 ± 5.0 | 30.4 ± 13.0 |

ing from infection with atypical *A. salmonicida* in goldfish. As such, our findings lend support to the previously stated view that vaccines containing atypical *A. salmonicida* cells may be beneficial when confronting disease attributed to these strains, insofar as commercial products developed with typical strains for salmonids are generally not effective (Lund et al. 2002). Certainly, there are indications that vaccines prepared with atypical isolates of *A. salmonicida* are successful; for example, the RPS of about 90% achieved in Atlantic halibut and spotted wolffish (Ingilae et al. 2000) is compatible with that achieved for ornamental fish in this study. Thus, there are parallels with the perceived benefits of autogenous vaccines (Gudmundsdóttir et al. 2003), in this case containing atypical *A. salmonicida* from the actual host. Similar to previous studies, our data point to the value of virulent (i.e., rough) cells of *A. salmonicida* for conferring greater protection than the smooth variant (Lund et al. 2003). Indeed, the A-layer of an atypical isolate of *A. salmonicida* has been determined to be immunogenic for goldfish (Sinyakov et al. 2001). However, the most successful vaccination regime involved cells of atypical *A. salmonicida* grown in conditions of iron restriction and iron supplementation together with cells of *A. bestiarum* grown on standard bacteriological medium (e.g., TSB) administered by an initial immersion followed by an oral booster. The approach is in agreement with previous studies with salmonids, when protection was enhanced after administration of an oral booster (Thorburn and Jansson 1988; Midtlyng et al. 1996). Moreover, Arijo et al. (2003) reported significantly increased protection in sea bream after booster vaccination, which they attributed to increased levels of antibody against bacterial lipopolysaccharides. Furthermore, the value of oral vaccination for stimulation of specific antibody production and innate cellular immunity has been well recognized (e.g., Durbin et al. 1999).

*Aeromonas bestiarum*, a recent addition to the range of fish pathogens (Kozinska and Guz 2004), may be beneficial as a component of the vaccine because of a possible immunostimulatory role, such as by means of lipopolysaccharide (Loghothetis and Austin 1996) and the presence of shared antigens with other bacterial species (Swain et al. 2003). Indeed, polyvalent vaccines have shown greater protection against *A. salmonicida* infections in Atlantic salmon than have monovalent preparations (Hoel et al. 1998; Lund et al. 2003).

The nature of the protection may well involve antibody production (Bricknell et al. 1999) and stimulation of innate immunity (Irianto and Austin 2002). Other workers have pointed to a greater correlation between protection and antibody titer (Bricknell et al. 1999) than we inferred in this investigation. However, any specific antibody response may take many weeks to develop and lead to protection, whereas innate mechanisms are activated within days (Ellis 2001). Thus, innate defense mechanisms might well be more important than previously realized. In this study, we found a statistically significant difference between vaccinated fish and controls regarding the number of head kidney macrophages absorbing trypan blue. Furthermore, although not statistically significant, the vaccinated fish had an increase in the number of leukocytes compared with the controls, which agrees with earlier work on probiotics (Irianto and Austin 2003).

In conclusion, the use of a polyvalent vaccine by immersion and an oral booster can protect goldfish against ulcer disease. However, it remains for further work to determine the precise duration of protection.

## Acknowledgments

Financial support from a DEFRA-Link Aquaculture Project (FC1143) is gratefully acknowledged.

## References

Ali, A., A. M. Carnahan, M. Altwegg, J. Luthy Hottenstein, and S. W. Joseph. 1996. *Aeromonas bestiarum* sp. nov. (formerly genomospecies DNA group 2 *A. hydrophila*), a new species isolated from nonhuman sources. Medical Microbiology Letters 5:156–165, 1996.

Amend, D. F. 1981. Potency testing of fish vaccines. Developments in Biological Standardization 49: 447–454.

Arijo, S., C. Balebona, E. Martinez-Manzanares, and M. A. Moriñigo. 2003. Immune response of gilt-head seabream (*Sparus aurata*) to antigens from *Photobacterium damselae* subsp. *piscicida*. Fish and Shellfish Immunology 16:65–70.

Austin, B., and D. A. Austin. 1989. Methods for the microbiological examination of fish and shellfish. Ellis Horwood, Chichester, UK.

Austin B., and D. A. Austin. 1999. Bacterial fish pathogens: disease in farmed and wild fish, 3rd (revised) edition. Springer-Praxis, Godalming, UK.

Austin, B., D. A. Austin, I. Dalsgaard, B. K. Gudmundsdóttir, S. Høie, J. M. Thornton, J. L. Larsen, B. O'Hici, and R. Powell. 1998. Characterization of atypical *Aeromonas salmonicida* by different methods. Systematic and Applied Microbiology 21: 50–64.

Bricknell, I. R., J. A. King, T. J. Bowden, and A. E. Ellis. 1999. Duration of protective antibodies, and the correlation with protection in Atlantic salmon (*Salmo salar* L.), following vaccination with an *Aeromonas salmonicida* vaccine containing iron-regulated outer membrane proteins and secretory polysaccharide. Fish and Shellfish Immunology 9: 139–151.

Duff, D. C. B. 1942. The oral immunization of trout against *Bacterium salmonicida*. Journal of Immunology 44:87–94.

Durbin, M., D. McIntosh, P. D. Smith, R. Wardle, and B. Austin. 1999. Immunization against furunculosis in rainbow trout with iron-regulated outer membrane protein vaccines: relative efficacy of immersion, oral, and injection delivery. Journal of Aquatic Animal Health 11:68–75.

Ellis, A. E. 2001. Innate host defense mechanisms of fish against viruses and bacteria. Developmental and Comparative Immunology 25:827–839.

Gudmundsdóttir, S., S. Lange, B. Magnadóttir, and B. K. Gudmundsdóttir. 2003. Protection against atypical furunculosis in Atlantic halibut, *Hippoglossus hippoglossus* (L.): comparison of a commercial furunculosis vaccine and an autogenous vaccine. Journal of Fish Diseases 26:331–338.

Hoel, K., L. J. Reitan, and A. Lillehaug. 1998. Immunological cross reactions between *Aeromonas salmonicida* and *Vibrio salmonicida* in Atlantic salmon (*Salmo salar* L.) and rabbit. Fish and Shellfish Immunology 8:171–182.

Ingilae, M., J. A. Arnesen, V. Lund, and G. Eggset. 2000. Vaccination of Atlantic halibut *Hippoglossus hippoglossus* L., and spotted wolffish *Anarhichas minor* L., against atypical *Aeromonas salmonicida*. Aquaculture 183:31–44.

Irianto, A., and B. Austin. 2002. Use of probiotics to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases 25: 332–342.

Irianto, A., and B. Austin. 2003. Use of dead probiotic cells to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases 26:59–62.

Kozinska, A., and L. Guz. 2004. The effect of various *Aeromonas bestiarum* vaccines on nonspecific immune parameters and protection of carp (*Cyprinus carpio* L.). Fish and Shellfish Immunology 16:437–445.

Loghothetis, P. N., and B. Austin. 1996. Antibody responses of rainbow trout *Oncorhynchus mykiss*, Walbaum to live *Aeromonas hydrophila* as assessed by various antigen preparations. Fish and Shellfish Immunology 6:455–464.

Lund, V., J. A. Arnesen, and G. Eggset. 2002. Vaccine development for atypical furunculosis in spotted wolffish *Anarhichas minor* O.: comparison of efficacy of vaccines containing different strains of atypical *Aeromonas salmonicida*. Aquaculture 204:33–44.

Lund, V., J. A. Arnesen, D. Coucheron, K. Modalsli, and C. Syvertsen. 2003. The *Aeromonas salmonicida* A-layer protein is an important protective antigen in oil-adjuvanted vaccines. Fish and Shellfish Immunology 15:367–372.

Midtlyng, P. J., L. R. Reitan, A. Lillehaug, and A. Ramstad. 1996. Protection, immune responses, and side effects in Atlantic salmon (*Salmo salar* L.) vaccinated against furunculosis by different procedures. Fish and Shellfish Immunology 6:599–613.

Sinyakov, M., M. Dror, S. Margel, and R. R. Avtallon. 2001. Immunogenicity of *Aeromonas salmonicida* A-protein in goldfish (*Carassius auratus* L.). Israeli Journal of Aquaculture Bamidgeh 53:110–114.

Steele, R. G. D., and J. M. Torrie. 1981. Principles and procedures of statistics. McGraw Hill, London.

Swain, P., S. K. Nayaka, A. Sahua, P. K. Meherb, and B. K. Mishra. 2003. High antigenic cross-reaction among the bacterial species responsible for diseases of cultured freshwater fishes and strategies to overcome it for specific serodiagnosis. Comparative Immunology, Microbiology, and Infectious Diseases 26:199–211.

Thorburn, M. A., and E. L. K. Jansson. 1988. The effects of booster vaccination and fish size on survival and antibody production following *Vibrio* infection of bath-vaccinated rainbow trout, *Salmo gairdneri*. Aquaculture 71:285–291.