General enquiries on this form should be made to:
Defra, Science Directorate, Management Support and Finance Team,
Telephone No. 020 7238 1612
E-mail: research.competitions@defra.gsi.gov.uk

defra
Department for Environment Food and Rural Affairs

SID 5 Research Project Final Report

- **Note**
  In line with the Freedom of Information Act 2000, Defra aims to place the results of its completed research projects in the public domain wherever possible. The SID 5 (Research Project Final Report) is designed to capture the information on the results and outputs of Defra-funded research in a format that is easily publishable through the Defra website. A SID 5 must be completed for all projects.
- This form is in Word format and the boxes may be expanded or reduced, as appropriate.
- **ACCESS TO INFORMATION**
  The information collected on this form will be stored electronically and may be sent to any part of Defra, or to individual researchers or organisations outside Defra for the purposes of reviewing the project. Defra may also disclose the information to any outside organisation acting as an agent authorised by Defra to process final research reports on its behalf. Defra intends to publish this form on its website, unless there are strong reasons not to, which fully comply with exemptions under the Environmental Information Regulations or the Freedom of Information Act 2000.
  Defra may be required to release information, including personal data and commercial information, on request under the Environmental Information Regulations or the Freedom of Information Act 2000. However, Defra will not permit any unwarranted breach of confidentiality or act in contravention of its obligations under the Data Protection Act 1998. Defra or its appointed agents may use the name, address or other details on your form to contact you in connection with occasional customer research aimed at improving the processes through which Defra works with its contractors.

Project identification

1. Defra Project code: AW1206
2. Project title: Fish welfare and health in sustainable aquaculture
3. Contractor organisation(s):
   Cefas Weymouth Laboratory
   Barrack Road
   The Nothe
   Weymouth
   Dorset
   DT4 8UB
4. Total Defra project costs (agreed fixed price): £ £194,528
5. Project: start date .... 01 October 2005
   end date .... 30 September 2008

6. It is Defra's intention to publish this form.
   Please confirm your agreement to do so. .... **YES** ☒ **NO** ☐

   (a) When preparing SID 5s contractors should bear in mind that Defra intends that they be made public. They should be written in a clear and concise manner and represent a full account of the research project which someone not closely associated with the project can follow.
   Defra recognises that in a small minority of cases there may be information, such as intellectual property or commercially confidential data, used in or generated by the research project, which should not be disclosed. In these cases, such information should be detailed in a separate annex (not to be published) so that the SID 5 can be placed in the public domain. Where it is impossible to complete the Final Report without including references to any sensitive or confidential data, the information should be included and section (b) completed. NB: only in exceptional circumstances will Defra expect contractors to give a "No" answer.
   In all cases, reasons for withholding information must be fully in line with exemptions under the Environmental Information Regulations or the Freedom of Information Act 2000.

   (b) If you have answered NO, please explain why the Final report should not be released into public domain

Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

- The welfare of fish is increasing in profile, and indicators are needed to assess the welfare status of farmed stocks. It is argued that welfare relates to the feelings of animals (in relation to the environment, husbandry conditions and practices to which they are exposed) and primary stress response indicators, being triggered by the brain, provide a gauge of negative feelings and therefore poor welfare.

- Cortisol, the classic stress hormone, is the most tractable primary stress indicator in fish with elevated levels in the blood indicating a response to a direct physicochemical or perceived threat. Traditional measurements of cortisol levels in the blood require invasive sampling and are therefore unsuitable for frequent use. Previously, we developed a non-invasive stress indicator for freshwater fish, based upon measuring cortisol release into the water via the gills, at a rate associated with the level in the blood. The current project was funded by Defra to further develop the methodology.

- Initial bench experiments illustrated that the non-invasive methodology (water sampling, concentration of cortisol from the water using solid phase extraction cartridges, retrieval by solvent elution, measurement by radioimmunoassay) previously validated for freshwater was transferable to seawater. A peer-review paper was published demonstrating the use of the non-invasive methodology for seawater Atlantic salmon. Two further review papers were written discussing the advantages, disadvantages and research requirements for non-invasive measurement of fish steroid hormones.

- The measured concentration of cortisol in the water depends not only upon the cortisol level in the fish, but also upon the number (and size) of the fish and the dilution (and removal) by inflowing water. Accurate information on fish biomass and water replacement is therefore needed to convert water cortisol concentration into "cortisol release rate" as an absolute measure of cortisol status. Fish biomass and water inflow rate are readily quantified in controlled laboratory tanks, but gaining accurate information is a barrier to transferring the methodology to commercial fish farms. The solution to the problem would be to measure an additional metabolite in the water that is excreted by fish at a consistent rate. Expressing water cortisol concentration as the cortisol: metabolite ratio would "normalise" values and provides an absolute measure of cortisol (stress) status. Within the project we examined creatinine and melatonin as candidate normalising metabolites by assessing release route and the effects of environmental factors on their excretion rate.

- In mammals, creatinine (a by-product of muscle physiology) is excreted at a consistent rate dependent

upon muscle mass and is used to normalise mammalian urinary cortisol concentrations for how dilute (or concentrated) urine samples are. We developed a method for measuring water creatinine concentrations in fish tanks, i.e. water sampling, creatinine concentration using cation exchange cartridges, retrieval by solvent elution, and measurement by photometric microplate assay. We demonstrated that fish excrete creatinine via the urine, as do mammals. Creatinine excretion rate was not affected by acute (handling) stress and showed a low level of variation (within and between tank), both qualities required of a normaliser. However, creatinine release rate was markedly affected by feeding rate and a change in tank conditions (which was thought to be due to a reduction in feeding rate). Furthermore uneaten fish food was shown to be a significant source of water creatinine, and it can therefore be assumed that excreted creatinine is of both endogenous and exogenous (food) origins. Creatinine was therefore shown to be unsuitable as a normaliser for water cortisol.

- Melatonin is a hormone released into the blood by the pineal in response to darkness. As it is not associated with the stress hormone axes, it had been suggested as a candidate normaliser. We had previously shown that the non-invasive method developed for cortisol was also suitable for measuring water melatonin. Within this project, we have demonstrated that melatonin release is not affected by acute (handling) stress. However, melatonin release was markedly affected by feeding rate and a change in tank conditions. Melatonin was therefore shown to be unsuitable as a normaliser for water cortisol.

- Although both candidate metabolites were shown to be unsuitable as normalisers for water cortisol (which prevents them being used to provide an absolute measure of stress status), there is nevertheless an alternative procedure that could be applied on fish farms – viz. making water cortisol measurements on a regular basis for use as a relative measure of stress status, i.e. assessing changes over time.

- Our previous work had shown water cortisol surges occurring during three disease challenges. Within the current project, we water sampled seven more disease challenge experiments and reviewed the published literature (32 studies) on fish cortisol responses to infections. Our sampling and review illustrated that cortisol responses can occur in fish during infections from viral, bacterial, fungal and parasitic pathogens, but only in those infections in which there is significant tissue damage, moribundity and mortality. Monitoring of water cortisol could therefore provide an early warning of severe disease outbreaks in farmed fish stocks [**if** a rapid, real-time measurement method was available].

- We assessed the future potential for developing a “dip-stick” or biosensor method for providing rapid, real-time measurements of water cortisol concentration. Nothing is currently available that is suitable for the ng/L cortisol concentrations typically found in fish water samples. However, by combining existing biosensor technology with recent developments in ultra-sensitive immunoassays, we believe that development of a cortisol biosensor is a realistic target for R&D.

- The non-invasive cortisol assay illustrated a cortisol response that lasted for several days in rainbow trout after transfer from the stock to experimental tanks - much longer than the recovery time from a handling stress. This illustrates that the change in tank conditions (but not water chemistry) is in itself a stressor and is attributed to acclimation. Elevated cortisol levels during acclimation were associated with reduced melatonin and creatinine release rates, further indicating physiological disturbance.

- Two separate experiments showed that the physiological disturbance (in cortisol, melatonin and creatinine) associated with acclimation was greater at a lower density (20 kg/m^3) than at a higher density (80 kg/m^3). Further research is needed to explore the possible causes and whether the effect occurs in commercial scale systems. Nevertheless, the finding does illustrate that the impact of density on welfare is more complicated than the simple assumption of a need for space.

- The experimental work demonstrated that short term food deprivation of 1 to 3 days can have a marked effect on physiological indicators. Food deprivation was associated with elevated cortisol levels at certain times of the day and a halving of night-time melatonin levels. It is generally assumed that such short periods of food deprivation in farmed fish are natural, do not affect their nutritional status (as poikilotherms) and are therefore of little concern for welfare. This assumption may therefore require re-examination, given the industry practice of food restriction prior to handling and slaughter, and for controlling growth rate.

- This project has demonstrated the potential that measuring excreted metabolites holds as a non-invasive means of monitoring the physiological state, health and welfare of farmed fish, as well as illustrating potential methodological and interpretational pitfalls of the approach. We foresee that, with further research, development and novel thinking, the measurement of metabolites in the water will become more commonplace for assessing the health and welfare of farmed fish.

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the scientific objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Transfer).

Introduction
The welfare of farmed fish has increased in profile since the early 1990s (**1**) and it is recognised that objective measures/indicators are needed to evaluate fish welfare status (**2**). This project was funded to further develop a **non-invasive stress assay for fish** as an objective fish welfare indicator - based upon measuring the release of cortisol (the classic stress hormone) into the water.

Animal welfare, feelings and stress
A typical definition of animal welfare is "the welfare of an animal is its state as regards its attempts to cope with its environment" (**3**). Such a definition may disguise the fact that it is the **feelings** of animals (in relation to the environment, husbandry conditions and practices to which they are exposed) that is the essence of animal welfare. Only those higher taxa and developmental stages of animals that are considered to have evolved the capacity for feelings (variously termed conscious, sentient, aware; **4-6**), are endowed with welfare consideration. The function and behaviour of lower animals (and plants) do change in relation to the environmental quality, but the deemed lack of a capacity for feelings means that these species are not bestowed with welfare consideration. The "Five Freedoms" (**7**), a well-respected foundation of animal welfare thinking, is simply a paraphrasing of negative feelings (e.g. freedom from feelings of hunger, thirst, pain, fear, discomfort, frustration, boredom etc). These freedoms are used to guide judgements on welfare, with an infringement of a freedom indicating the possible presence of negative feelings, and hence suffering and poor welfare. Thinking has moved on since the conception of the "Five Freedoms" and now, rather than being considered as a binary state (i.e. if welfare is not poor then it is good), animal welfare is considered as a continuum:

- poor welfare is associated with negative feelings (e.g. pain, hunger, fear, illness, sadness)
- neutral welfare is associated with no negative and no positive feelings (or a net balance)
- good welfare is associated with positive feelings (e.g. happiness) (**10**).

Feelings (how does the animal feel?) may appear to be only one of three approaches to viewing and assessing animal welfare (**9**), the other two being *function* (is the animal able to function well?) and *behaviour* (is the animal behaving naturally/normally?). These two alternative approaches recognise the fact that the objective assessment of an animal's (subjective) feelings is an extremely difficult task, and are preferred by those uncomfortable with attributing feelings to non-human animals (**10**). However, these two alternative approaches to animal welfare do have feelings at their core due to the inherent assumptions:

- If an animal cannot function well then negative feelings may ensue and vice-versa, if an animal has negative feelings this may express as compromised function
- If an animal cannot perform natural behaviour then negative feelings may ensue and vice-versa, if an animal has negative feelings this may be expressed as abnormal or compromised behaviour.

Gauging the subjective feelings of animals is recognised to be problematic. The most objective means available for assessing the presence of negative feelings in animals is acknowledged to be the measurement of indicators of stress status (**11**). The underlying assumption is that negative feelings in animals will manifest in a physiological stress response, analogous to that which we accept occurs in humans (**12,13**). Stress is therefore a key issue within the field of animal welfare, and measures of stress status are a valuable indicator of animal welfare.

A stress response in fish can be measured at different levels of response (**14-17**):

- 1° response - the hormonal responses – the perception of a threat by the brain triggers release of stress hormones (catecholamines and corticosteroids, e.g. adrenalin, cortisol) that precipitate the 2° response.
- 2° response - the physiological changes (e.g. increased number of red blood cells, increased glucose in blood) and behavioural changes (e.g. reduced feeding activity) that enable the animal to respond to the threat. However, if these 2° responses are maintained over a period of time, 3° responses can result.
- 3° responses – the effects at the whole animal level, e.g. decreased growth and reproductive investment, increased disease susceptibility and mortality.

These three levels of response parallel the stages of Alarm, Resistance and Exhaustion in Seyle's classic model of the Generalised Adaptation Syndrome.

If animal welfare is viewed solely from a functional perspective then it would be most appropriate to measure the 3° (and possibly 2°) stress responses, as such measures indicate how the animal is coping with its environment. However, if we are trying to get a handle on how an animal perceives (and feels about) the environment, then the 1° response is the most appropriate measure as it is directly triggered by the brain. The perennial problem with such measures (and any indicator of welfare) is that it cannot be known if these truly do indicate the occurrence of negative feelings (psychological stress), or they are simply due to physiological processes of which the animal is mentally unaware.

The stress hormone cortisol and fish welfare assessment

Some scientists have argued that fish do not have the mental capacity for feelings (due to the lack of a cortex region in the brain) (**18,19**) and therefore do not merit welfare consideration. Others have accepted the concept of fish welfare but dismissed the consideration of fishes' feelings (**10**). Fish have the very same stress hormones (adrenalin and cortisol) as mammals, and a review of fish cortisol studies has shown that fish do perceive a wide variety of indirect (visual, chemical, acoustic, learnt/conditioned) threats and exhibit a cortisol response (**20**). Cortisol responses therefore indicate that fish posses a psychological capacity that supports the concepts that they

- have an awareness of the environment similar to that of mammals and birds (**5**) which are generally accepted to have feelings and merit welfare consideration
- they may experience negative feelings

Cortisol, as an indicator of stress and potentially feelings is, arguably, the most valuable gauge of the welfare of farmed fish. Cortisol has been measured, almost ubiquitously, in recent fish welfare studies (**21-23**). Inherent in traditional measurements of the concentration of cortisol in the blood (elevated levels indicating stress) of fish is that the method requires invasive sampling that disturbs both the sampled and co-habitant fish. The analogous problem in birds and mammals led to the development of non-invasive methods for assessing cortisol excreted in urine, faeces and saliva (**24**). With previous support from Defra (Projects AW1203, FC0916) we have developed and validated a non-invasive procedure for fish, based upon measuring the release of cortisol into the water. The method allows populations of fish to be sampled without disturbance or sacrifice. We have applied the methodology in tank experiments to assess the stress associated with handling, confinement, tagging (**25-27**) and stocking density (**28,29**), and it has been taken up by the wider scientific community (**30,31**).

Our non-invasive cortisol assay for fish is based upon measuring the concentration of cortisol in the water (instead of the blood). Obviously the water cortisol concentration will depend not only upon how fast it is released by the fish, but also upon the number (and size) of the fish and the dilution and removal by inflowing water: this information is therefore needed to convert water cortisol concentration into "cortisol release rate" as an absolute measure of cortisol status (**25**). Fish biomass and water inflow rate are readily quantified in controlled laboratory tanks, but gaining accurate information on biomass and water exchange rates was found to be a barrier to transferring the methodology to commercial rainbow trout farms (**28,29**). It was also foreseen as a major problem in applying the method to sea cages, where the exposure of a water sample to the fish would be unknown.

For mammals, non-invasive measurement of cortisol in urine faces a similar problem – i.e. adjusting the cortisol measurement to take account of the dilution of the urine. The problem has been overcome by measuring an additional compound (creatinine) in the urine that is excreted at a consistent rate. Expressing urinary cortisol as the cortisol: creatinine ratio therefore "normalises" values and provides an absolute measure of cortisol (stress) status (e.g. **32**).

The project

This project was funded by Defra to further develop the non-invasive cortisol assay for fish and "match-fund" Cefas's commitment to the EU FP6 WEALTH project (Welfare and Health in Sustainable Aquaculture, hence the cryptic project title of AW1206). The scientific aims of the project were to:

- Complete the validation of the measurement of cortisol and melatonin in seawater (so that the non-invasive methodology could be applied in tank experiments on salmon and sea-bass within the WEALTH project).
- Conduct tank experiments to examine the potential of two candidate "normalisers" for cortisol, i.e. melatonin and creatinine - to ultimately facilitate interpretation of cortisol concentrations in water samples from fish farms.

- Sample disease challenge experiments at Cefas Weymouth to assess the potential for water cortisol to provide an early warning of disease outbreaks.
- Examine the possibility of developing a "dip-stick" test for cortisol which would enable simple, real-time assessment of cortisol as an indicator of stress and disease (fish welfare) status.

These objectives were met as detailed below.

Validation of measurement of cortisol and melatonin in seawater

The methodology for non-invasive measurement of cortisol involves collection of water samples, concentration of the cortisol from the water by passing through a solid phase extraction cartridge (SPEC) containing an absorptive matrix of C18, retrieval of the cortisol from the SPEC by elution with an organic solvent, evaporation of the solvent, dissolving the residue (containing the cortisol) in an appropriate buffer, and finally measurement by radioimmunoassay.

Initial work addressed concerns that this extraction methodology previously validated for cortisol (**25**) was not optimal for melatonin (**33**). Laboratory experiments indicated that the solvent (ethyl-acetate) found appropriate for cortisol caused variable recovery of melatonin due to oxidation: the magnitude of the problem was exaggerated when the solvent was evaporated in air rather than under nitrogen. Alternative solvents were trialled, but we opted to continue with ethyl-acetate but incorporate an antioxidant (resveratrol: 0.005 mg/mL ethyl-acetate). This revised method allowed cortisol and melatonin to be measured within the same sample extract.

Initial bench experiments conducted within this project aimed to validate the measurement of cortisol and melatonin in seawater by examining

- recovery of cortisol and melatonin "spikes" added to water samples collected from a stock tank of seawater salmon
- the stability of cortisol and melatonin in seawater samples over time, and the possible effect of oxygenation
- the stability of cortisol and melatonin during freeze storage of seawater samples and SPECs

These background studies demonstrated that the non-invasive methodology - measuring cortisol and melatonin levels in water samples – was transferable from freshwater to seawater. Both hormones were secreted into seawater by Atlantic salmon and could be extracted and measured. Supplemental oxygenation of water (an increasingly common aquacultural practice to safeguard fish held at higher temperatures and densities) resulting in supersaturated oxygen levels did not reduce the recovery of cortisol from seawater. (It had previously been suggested that elevated oxygen levels might degrade (oxidize) the cortisol). The non-invasive method was therefore shown to be suitable for seawater. This work has been presented at two international conferences, and published as a paper integrating the above experiments with previous work (under FC0916 and the WEALTH project) on the release of cortisol and melatonin by seawater salmon (**34**). Two further review papers have been written/co-authored and published discussing the usage, advantages and disadvantages, and further work required for non-invasive assays for fish steroids (**30,31**). All these papers have highlighted the need for a normaliser to facilitate interpretation of water steroid levels. Within this project we examined creatinine and melatonin as potential normalisers.

Development of methodology for measuring creatinine

A lot of research has been conducted on creatinine in mammals (**35**). Creatinine is a spontaneously formed cyclic breakdown product of creatine phosphate. Creatine phosphate is involved in supplying energy to muscle tissue and the production of creatinine is, therefore, largely proportional to muscle mass and is relatively independent of activity. Creatinine is primarily non-selectively filtered out of the blood, into the urine, by the kidneys with negligible tubular reabsorption (**35**). Consequently, production and excretion of creatinine in mammals is mainly dependant on muscle mass and this property is exploited by using the urinary creatinine concentration to adjust urinary cortisol concentrations for how dilute (or concentrated) the urine is, i.e. by using the cortisol: creatinine ratio (**32**). In contrast to mammals, little information is available on creatinine production and excretion by fish. However, fish do excrete creatinine and it is assumed to play an analogous role in muscle physiology to that in mammals (**36-39**).

Methods for measuring creatinine in mammalian urine are well established and commercial colorimetric microplate assays are readily available. We compared seven commercially available assay kits. All the assays measured creatinine over a similar range of concentrations based upon the same methodology – the "Jaffe reaction" causes a measurable colour change in relation to creatinine concentration. However, only two of the kits employed an additional acidification stage to eliminate cross-reaction with other compounds. Due to the potential for interfering compounds in water samples, we opted for a two-stage assay (Oxford Biomedical Research) and used it throughout the project. This microplate kit measures creatinine within the range 1-10 mg/dL (≡ 10-100 mg/L) in 25 µL of sample, and initial trials proved that the kit was simple to use and the standard curve remained linear at lower creatinine concentrations (≥1 mg/L).

We then predicted likely water creatinine concentrations in fish tank water samples using the limited existing literature on creatinine excretion rates by fish (**39**) which indicated that creatinine concentrations would be too low to measure directly and would need to be concentrated by a factor of 1000 for measurement (**Table 1**). Solid-phase extraction (already used for concentrating cortisol and melatonin from water samples) was identified as a suitable concentration methodology. It was shown that the C18 absorbent matrix (Sep-pak®) used for steroid hormones was unsuitable, but a cation exchange matrix was identified as suitable (**40**) and an appropriate product was sourced (Waters Oasis® MCX). Initial trials demonstrated that recovery of spiked creatinine from distilled water was high (ca. 100%), and that naturally excreted creatinine could be concentrated from fish tanks and measured readily.

Table 1: Prediction of likely creatinine concentration in water samples from fish farms

Measure	**Unit**	**Value**	**Source**
Excretion rate	nmol N/g/h	11.1	(**39**)
Excretion rate	nmol/g/h	3.7	÷ by 3 to convert N to creatinine ($C_4H_7N_3O$)
Excretion rate	ng/g/h	418	x by 113 to convert nmol to ng (MW creatinine =113)
Excretion rate	ng/kg/min	6968	x 1000 (convert /g to / Kg); ÷ 60 (convert /h to /min)
Excretion rate	mg/kg/min	0.007	÷ 1000000 (convert ng to mg)
Biomass loading rate	kg/L/min	1	ballpark value based on previous experience (**28**)
Expected creatinine concentration	mg/L	0.007	mg/Kg/min x kg/L/min

To optimise the cation exchange extraction method, the water samples need to be acidified (**41**). Trials demonstrated that 25 ml/L sodium acetate buffer (pH 5) was suitable. Then, when moving from distilled water to spiked "real" water samples, we identified a recovery problem: when creatinine spiked water samples (1L) were processed using 225 mg MCX SPEC, recovery was much reduced from 100% in distilled water to 50% in freshwater (dechlorinated Weymouth mains supply; pH 7.9; alkalinity 233 mg/L $CaCO_3$) and 15% in natural seawater (sand-filtered 35‰). It was then shown that the recovery of creatinine was inversely proportional to the volume of water that was processed, and this effect was more marked in seawater than in hard freshwater (**Fig 1A**). Consultation with the supplier confirmed that although MCX SPEC are designed for biological fluids, natural salts in samples do affect adsorption by the cation exchange matrix. The solution to the problem was to use SPEC containing a larger amount of matrix. We conducted trials demonstrating that use of appropriately larger sized MCX SPEC (500 mg for freshwater; 1000 mg for seawater) enabled recoveries of ≈90% for 0.25 L water samples (**Fig 1B-D**).

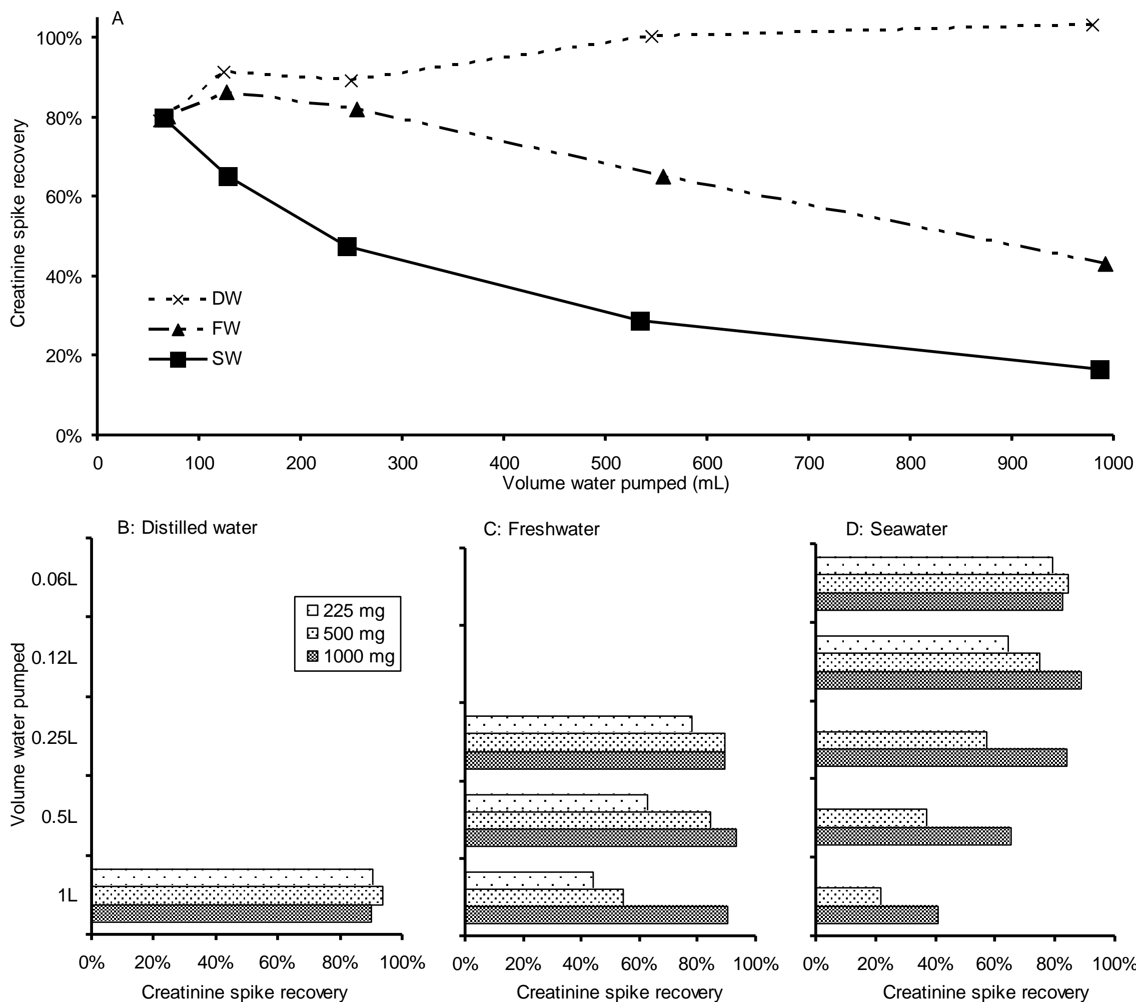

Figure 1: A: Effects of water type (DW=distilled water, FW=freshwater, SW=seawater) and volume of water sample processed on recovery of spiked creatinine. B-D: Effects of volume of water sample processed (0.06, 0.12, 0.25, 0.5, 1 L) and amount of matrix in cation exchange SPEC (225, 500, 1000 mg) on recovery of spiked creatinine from distilled water (B), hard freshwater (C) and seawater (D). n=2 per data point.

The solid-phase extraction and assay methods adopted for creatinine are compared to those established for the hormones (cortisol and melatonin) in **Table 2**.

Table 2: Comparison of stages for the concentration and measurement of metabolites in fish tank water samples.

Stage		for cortisol and melatonin	for creatinine
Solid-phase extraction cartridge for concentration of metabolite	SPEC	Solid-phase C18 – Sep-pak, 225 mg	Cation Exchange – Oasis MCX, 500 mg for freshwater
	Prime/Activate	Methanol (10 mL)	Methanol (10 mL)
	Wash	Distilled water (10 mL)	Distilled water (10 mL)
	Load	Pump 0.5 – 1 L sample	Pump 0.25 L acidified sample (25 mL/L sodium acetate buffer, pH 5)
	Wash 1	Distilled water (5 mL)	2% Formic acid (10 mL)
	Wash 2		Methanol (10 mL)
	Elute	Ethyl-acetate (5 mL) with 0.005 mg/mL resveratrol	Methanol with 2% NH_4OH (10 mL)
Solvent evaporation		Under nitrogen at 45°C	Under nitrogen at 45°C
Reconstitute		Radioimmunoassay (RIA) buffer- amount depending upon expected hormone concentrations, typically 1000 μL	Distilled water –amount dependant upon expected creatinine concentration, typically 125 μL
Measurement		RIA of 100 μL aliquots	Plate photometric assay of 25 μL aliquots

Description of tank experiments with rainbow trout to examine potential of creatinine and melatonin as normalisers

Four experiments were conducted with live rainbow trout (*Oncorhynchus mykiss*) within the Cefas Weymouth Experimental Facility (methods and results are detailed below). The overall aim of these experiments was to assess the suitability of creatinine and melatonin to normalise water cortisol concentrations for fish biomass and flow rate. These experiments examined the release routes and the lability of the candidate normalisers to the environmental factors of acute stress, density, food supply and temperature. The experiments also provided additional information on the impact of these factors on fish physiology.

During experiments, fish were fed appropriate sized commercial pellets three times daily "to table", i.e. at the ration recommended by the feed manufacturer for the fish size and temperature. Fish were maintained on a 12 h light: 12 h dark photoperiod, with a dawn and dusk fade. Timing of the photoperiod was controlled so that water samples could be collected just before the onset of the light phase in the morning of the working day: this was to facilitate sampling for melatonin. Tank water samples were collected via a permanent siphon tube (with tap) without disturbing the fish. Water cortisol, melatonin and creatinine concentrations were measured as described above. In addition, ammonia (the main nitrogenous metabolite excreted by fish) was measured directly in water samples using a commercial colorimetric method (Palintest® reagents and photometer).

Metabolite release rates (ng/g/h) were calculated by two different methods, dependent upon whether release was assumed to be stable (and hence in equilibrium with removal rate), or to be changing rapidly over time (**28**). The latter calculation method requires two measurements of metabolite concentration at different times, and the release rate over the period is calculated from the change in metabolite concentration, allowing for dilution by inflowing water. Within each experiment, some water samples were spiked with known amounts of standard cortisol, melatonin and creatinine to confirm recovery efficiency for each metabolite.

All experiments were conducted under appropriate Home Office (Animal Scientific Procedures 1986 Act) project and personal licences (Objective 2) and were subject to approval by the in-house Local Ethical Review Process Committee and statistician. Fish were humanely killed at termination of each experiment. Statistical analyses were by ANOVA (Stata™ 9.0), typically testing for the effects of treatment (i.e. density, food deprivation, temperature), stress (handling stress v unstressed control), sampling time (repeated measure), treatment*time interaction, and tank (nested within treatment) on cortisol, melatonin, creatinine and ammonia release rates. Variation in metabolite release rate (between replicates, between samples of the same tank over time) was quantified as the Coefficient Of Variation (COV= standard deviation/mean) expressed as %.

Experiment 1: Release route of creatinine

The objective of this experiment was

- to determine the route whereby fish excrete creatinine into the water.

Fish can release metabolites into the water via the skin, gills, urine and faeces. We had previously demonstrated that fish release cortisol (free, unconjugated) and melatonin into the water via the gills using a specially constructed tank (**27**). We adopted an identical procedure for the present experiment. Individual rainbow trout (n=6; mean mass ± S.E. 603 ± 4 g) were taken from a stock held at Cefas Weymouth and anaesthetised (125 μl/L phenoxy-ethanol solution) immediately prior to, and throughout, the experimental procedure. The fish were classed as lightly sedated (Stage I, Plane 1) - showing a normal opercular rate and voluntary swimming movements, but with some loss of reactivity to external stimuli (**42**). The fish were placed in a purpose-built 46 x 9 x 12.5 cm tank (marine plywood varnished with polyurethane) containing 3.5 L of freshwater (no flow through). A double latex diaphragm (dental rubber dam, Ash Instruments Division, Weybridge, UK) divided the tank into two sections at a point one third of the length. A hole (approx. 2 cm in diameter) was previously cut in the diaphragm. The fish was eased head first through this hole up to a point just behind the gills, with the pectoral fin bases preventing backward movement. The diaphragm prevented mixing of water between the anterior (head) and posterior (body/tail) sections (**Fig 2**). Aeration was supplied via diffuser stones to both anterior (c. 1.2 L) and posterior (c. 2.3 L) sections, and water temperature was maintained at 16°C by keeping the tank within a temperature controlled unit. Trials were conducted in the light and lasted ca. 2.5 h. At the end of each trial, it was recorded whether regurgitated food / faeces were absent or present in the anterior / posterior sections of the tank. The water was collected separately from the two sections and the actual volumes measured. The amounts of creatinine, cortisol and ammonia released into the anterior and posterior tank sections were calculated (concentration x water volume in each section), and release rates (ng/g/h) were further derived using the fish mass and trial duration.

Most of the cortisol (mean 98%) excreted was recovered from the anterior section of the tank (**Fig 2**), indicating release via the gills as previously documented (**27**). In contrast, most of the creatinine (mean 96%) was recovered from the posterior tank section. The absence of faeces in the trial tank indicates that the creatinine was excreted by the fish via the urine, i.e. the same route as in mammals. Most of the ammonia (89%) was recovered from the anterior end of the tank (**Fig 2**), confirming excretion via the gills as already widely accepted (**37**).

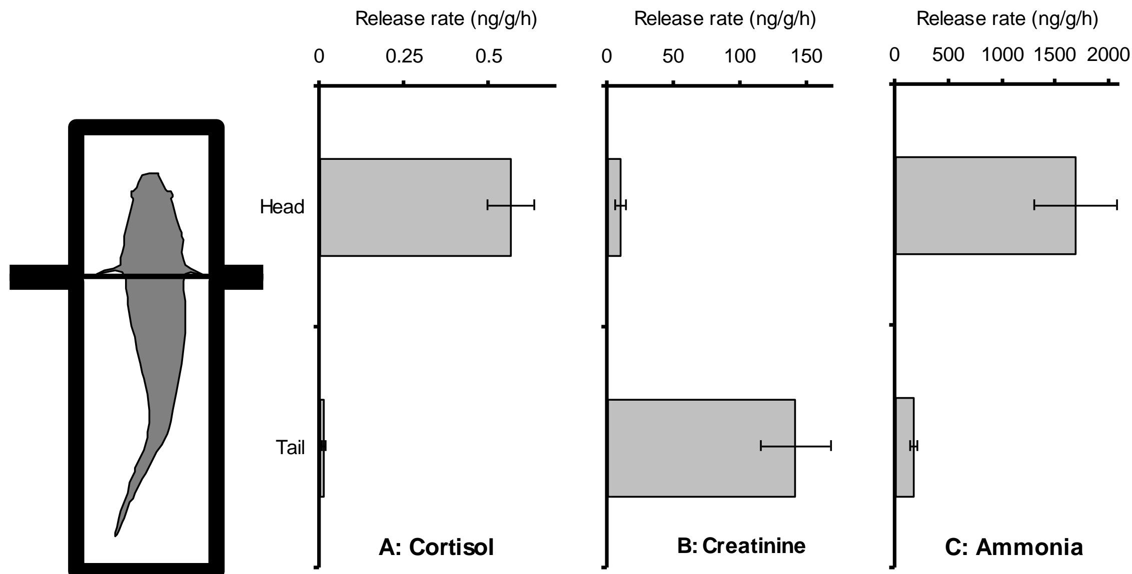

Figure 2: Release of cortisol (A), creatinine (B) and ammonia (C) into the water by the anterior and posterior sections of rainbow trout. Water was collected after ca. 2.5 h exposure to either the head or body/tail of individual fish, as indicated by image on left. Note changes in scale of horizontal (dependent variable) axes.

Experiment 2: Effects of stress and density on cortisol, melatonin and creatinine release
This experiment was conducted to examine whether:

- acute (handling) stress affects the release rates of cortisol, melatonin and creatinine by rainbow trout
- fish density affects the release rates of cortisol, melatonin and creatinine

The study was conducted in 12 tanks (30 L) supplied with fresh water (12°C) at a flow rate of 1.2 L/min/tank. Fish (39.3 ± 0.7 g; mean ± SEM) were stocked at 2 density treatments (20, 80 kg/m^3; 6 tanks per treatment). After a 2 week acclimation period, 3 tanks from each density treatment were subject to an acute handling stress (90 sec aerial emersion, **25**). The stress was applied 15 min before the start of the dark period to determine any potential effect of the stress on melatonin release (melatonin is only released during the dark, **33**). The experiment was therefore a 2 x 2 treatment design: 2 stocking densities (20 and 80 kg/m^3) and 2 stress treatments (stressed, unstressed control), with 3 replicates of each combination. In line with industry practice, the fish were not fed on the day of the handling stress. To examine the impact of the acute stress and density on metabolite release rates, water samples were collected:

- 0.5 h before and immediately prior to the stress
- 2.5 and 3 h after the stress
- 12 and 12.5 h after the stress
- 36.5 h after the stress

Cortisol: Cortisol release rate was affected by stress ($p<0.0001$) and time ($p<0.0001$) and the stress*time interaction ($p<0.0001$). The handling stress caused a typical cortisol stress response with cortisol release rate being elevated in the treatment tanks at the first two sampling times (midpoint 2.75, 12.25 h) after the handling stress, and decreasing to match the unstressed control fish at the 36.5 h sample (**Fig 3A**). There was no effect of density on the cortisol response to stress (density*stress interaction: $p>0.5$), although cortisol release rate was slightly higher in the 80 kg/m^3 tanks ($p<0.01$; **Fig 3A**).

Melatonin: Melatonin release rate was affected by time ($p<0.0001$), increasing from very low levels in the light period (-0.25 h) to higher values in the dark periods (**Fig 3B**). Melatonin release rate was not affected by the stress treatment ($p>0.5$) and there was no stress*density interaction ($p>0.5$). There was however an effect of density on melatonin release ($p<0.01$) which changed with time (density*time, $p<0.05$).

Creatinine: Creatinine release rate changed over time ($p<0.0001$), with the 36.5 h sample being greater than the previous samples (**Fig 3C**). The low creatinine release observed for the first 3 sample periods is thought due to the food deprivation (as explored later in Experiment 3) prior to application of the stress treatment. Creatinine release was not affected by stress ($p>0.5$) nor density ($p>0.1$), although there was a significant density*time interaction ($p<0.05$) with a higher release rate at 36.5 h at the higher density (**Fig 3C**).

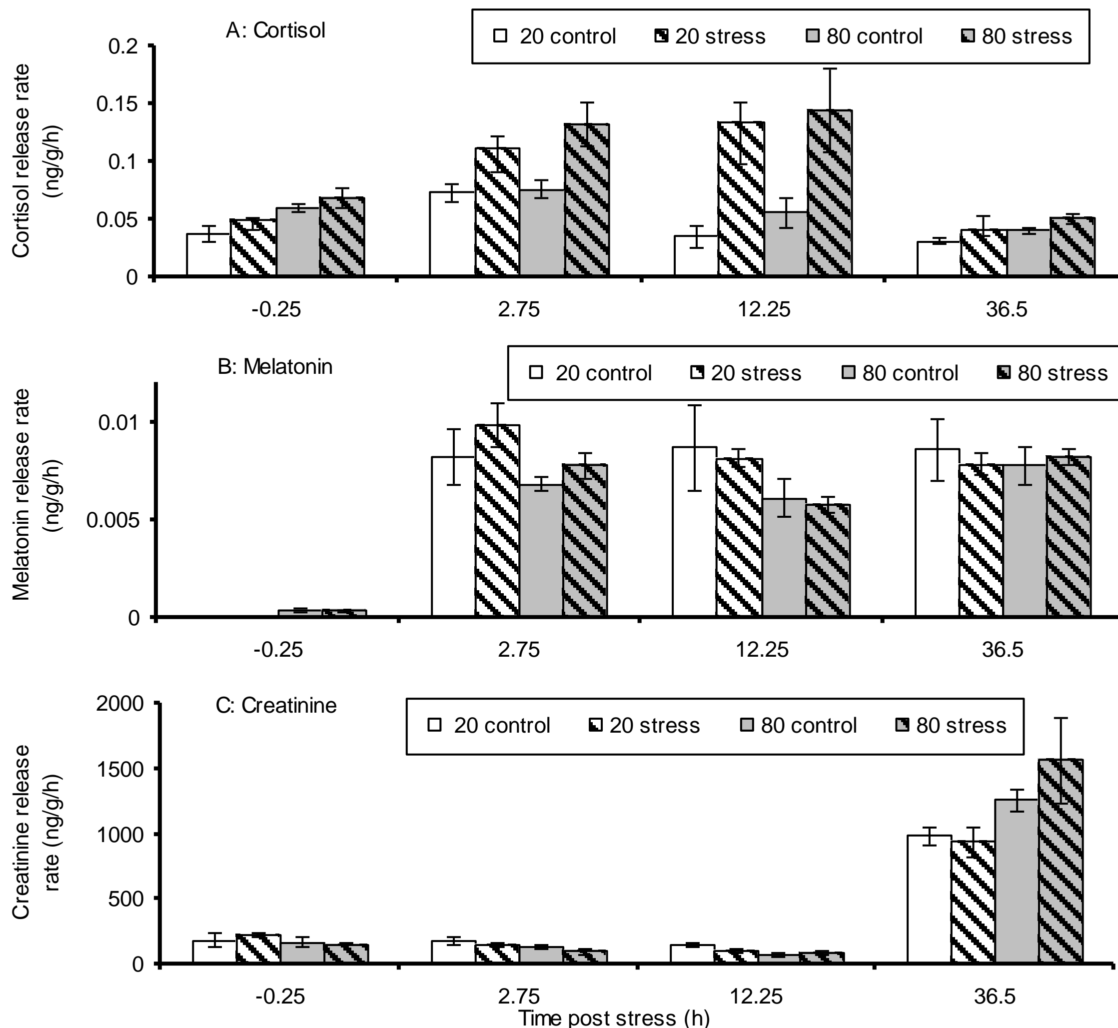

Figure 3: Release rates of cortisol (A), melatonin (B) and creatinine (C) at four different times in tanks of fish held at two different densities (20 & 80 kg/m^3) and either subjected to an acute handling stress, or left undisturbed (control). Values are means (±S.E, n=3 tanks per treatment combination).

In addition to the above sampling examining responses to handling stress, additional water samples were taken for analysis of cortisol, melatonin and creatinine:

- from the stock tank from which the experimental fish derived on 5 occasions prior to stocking (n=5 samples)
- from 6 experimental tanks at 08:00 (timed to coincide with the end of the dark period) on 8 days prior to the acute stress (n=48) to examine acclimation to the experimental tank conditions, the effect of density, and variation between replicate tanks
- from 6 experimental tanks at 3 h intervals over a 27 h period on days 9/10 after stocking (n=54) to examine variation over a diurnal cycle period and between replicate tanks

Cortisol: Cortisol release was stable in the stock tank prior to removal of the experimental fish (**Fig 4A**), was 5-fold higher in the experimental tanks on the day after stocking, and decreased to a rate comparable to that in the stock tank over the following week. This initial elevation of cortisol for the first few days after stocking is thought to represent acclimation to the experimental tank conditions (**34**). Cortisol release was higher at 20 than at 80 kg/m^3 on days 2 and 3 (density*day interaction: $p<0.05$). Over the 27 h cycle on days 9-10, there was no effect of time of day ($p>0.1$), but overall cortisol release rate was higher at the higher density ($p<0.05$; **Fig 4D**).

Melatonin: Melatonin release decreased markedly in the experimental tanks on the first 2 days after stocking from higher levels in the stock tank, followed by a subsequent increase (**Fig 4B**). Melatonin release was lower at 20 than at 80 kg/m^3 over the first week (density: $p<0.0001$, density*day interaction: $p<0.05$). Over the 27 h period, density did not affect melatonin release ($p>0.2$), but there was a marked effect of sampling time ($p<0.0001$): values dropped at the onset of light, remained negligible during the light period, and increased after the onset of darkness (**Fig 4E**).

Creatinine: Creatinine release showed a marked decrease in the stock tank prior to removal of the experimental fish (**Fig 4C**): this is thought to be due to food deprivation the previous day due to the imminent fish handling (see below). In the experimental tanks, creatinine levels were initially low but increased subsequently (**Fig 4C**). Again there was a difference between the two density treatments, being lower at 20 than at 80 kg/m^3 (density $p<0.0001$;

density*day interaction p=0.05; Fig 4C). Over the 27 h period, density did not affect creatinine release (p>0.25), and there was no significant effect of time (p>0.05) (Fig 4F). Within and between tank variation in creatinine release was typically lower for creatinine than for cortisol and melatonin (**Table 3**).

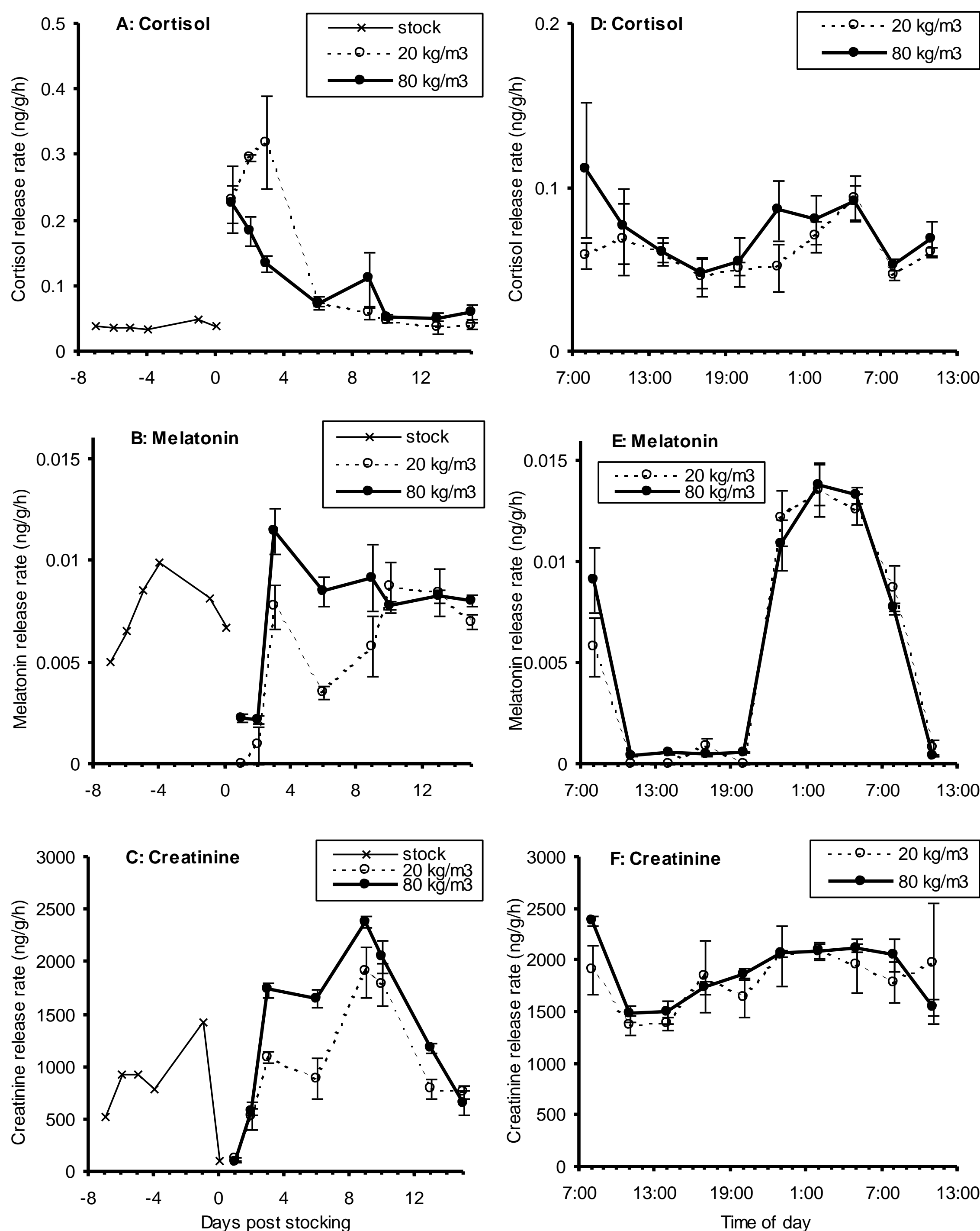

Figure 4: Time series of release rates of cortisol (A), melatonin (B) and creatinine (C) in stock tank and in experimental tanks (2 different densities: 20 & 80 kg/m^3) at 08:00 on various days prior to and after stocking the experimental tanks. Release rates of cortisol (D), melatonin (E) and creatinine (F) in experimental tanks (2 different densities: 20 & 80 kg/m^3) at various times on days 9 and 10 after stocking the experimental tanks. Values for experimental tanks are means (±S.E, n=3 tanks per density treatment).

Table 3: Within tank and between (replicate) tank variation (measured as coefficient of variation, COV) in metabolite release rates over the 27 h period. Values for melatonin were derived from values for dark period only.

	Mean COV within tank		Mean COV between tanks	
	20 kg/m^3	80 kg/m^3	20 kg/m^3	80 kg/m^3
Cortisol	30%	36%	28%	35%
Melatonin	34%	26%	23%	15%
Creatinine	15%	8%	22%	7%

Experiment 3: Effect of food deprivation on cortisol, melatonin and creatinine release
This experiment was conducted to examine whether

- food deprivation affects cortisol, melatonin and creatinine release rates
- food is a source of cortisol, melatonin and creatinine measured in tank water

The study was run in seven 30 L tanks supplied with fresh water (12°C) at a flow rate of 1 L/min. Rainbow trout (27.3 ± 0.3 g; mean ± SEM) were stocked at a density of 20 kg/m^3 into six of the tanks and acclimatised for 1 week before the start of the experimental phase. The study comprised one treatment (food deprivation for 3 days; 3 replicate tanks), a positive control (feeding continued as per acclimation; 3 replicate tanks), and a negative control (no fish but flow, cleaning and feeding in an identical manner to the positive control; 1 tank). After 3 days of food deprivation, the treatment fish were returned to the feeding regime. The fish were maintained for a further 2 days before the experiment was terminated.

Water samples were collected for assessment of water cortisol, melatonin and creatinine concentrations at 08:00 on days 1, 2, 3 and 6 during acclimation and days 7-10 during and after the food deprivation. Additionally, a 24 h series of samples (08:00, 11:00, 14:00, 17:00, 20:00, 23:00, 02:00, 04:00, 08:00) was collected over the third day of food deprivation. Water ammonia concentrations were measured over the 24 h cycle and during and after the food deprivation period. The results are illustrated as water metabolite concentrations (rather than release rates) to enable display of data for the no-fish tank. Statistical analysis was performed on the water metabolite concentrations to fit with the illustrations: as fish biomass and flow rates were matched, conclusions on water concentrations will equate to those for release rates. The data was split into three sets for analysis reflecting the different treatment periods and sampling times: 1) 08:00 samples on days 1, 2, 3 and 6 before food deprivation, 2) 08:00 samples on days 7, 8, 9 and 10, i.e. during and after food deprivation, 3) over the 24 h sampling period during food deprivation.

Cortisol: Prior to the food deprivation, there was no difference in cortisol release between the tanks destined for the treatment and the control tanks ($p>0.1$). There was however, a significant effect of sampling occasion ($p<0.0001$), with cortisol decreasing over the week after stocking (**Fig 5A**). Once the food deprivation started, there was no significant effect of this treatment on cortisol level in samples taken at 08:00 ($p>0.1$, food deprivation*day interaction, $p=0.06$), but a treatment effect was evident in the 24 h cycle of samples ($p<0.02$; food deprivation*day interaction, $p<0.05$). Cortisol levels were elevated in the food deprived tanks in samples collected at 11:00 and 14:00 (**Fig 5E**). Cortisol concentrations in the fed tank with no fish were either zero or negligible.

Melatonin: Prior to the food deprivation, there was a surprising difference in melatonin release between the tanks destined for the treatment and the control tanks ($p>0.0001$). The destined treatment tanks had higher melatonin release (**Fig 5B**), which is inexplicable as all tanks were treated the same at those time-points. Once the food deprivation started, the opposite difference was apparent with melatonin release being reduced by approximately half in the food-deprived tanks (**Fig 5B&F**) in samples taken at 08:00 ($p=0.0001$) and over the 24 h cycle ($p<0.0001$). No melatonin was recorded in samples from the fed no-fish tank.

Creatinine: Prior to the food deprivation, there was no difference in creatinine release between the tanks destined for the treatment and the control tanks ($p>0.5$). There was a significant effect of sampling occasion ($p<0.0001$), with creatinine release increasing after stocking (**Fig 5C**). Once the food deprivation started, there was a highly significant effect of this treatment on creatinine levels in samples taken at 08:00 ($p<0.0001$) and in the 24 h cycle samples ($p<0.0001$), with creatinine release being greatly reduced in the food-deprived tanks (**Fig 5C&G**). Low levels of creatinine were observed in samples from the fed tank with no fish collected at 08:00, and noteworthy concentrations were observed in this tank in the daytime samples at 11:00, 14:00 and 20:00. These creatinine peaks appear to correspond to the daily meals, given at approximately 09:00, 13:00 and 17:30.

Ammonia: Ammonia levels were very different in both series of samples taken during the food deprivation period ($p<0.0001$). Ammonia levels were around 6-fold lower in the food-deprived tanks (**Fig 5D&H**). Negligible levels of ammonia were recorded in the fed tank without fish.

Water concentrations of cortisol were typically more variable those of the other metabolites measured (**Table 4**). Melatonin (dark period samples only) were the least variable, while food deprivation appeared to increase the variability in both creatinine and ammonia concentrations.

Table 4: Within tank and between (replicate) tank variation (measured as coefficient of variation, COV) recorded over the 24 h period. Values for melatonin were derived from values for dark period only.

	Mean COV within tank		Mean COV between tanks	
	Fed	Food deprived	Fed	Food deprived
Cortisol	55%	42%	33%	36%
Melatonin	7%	6%	14%	18%
Creatinine	30%	64%	11%	61%
Ammonia	18%	36%	15%	24%

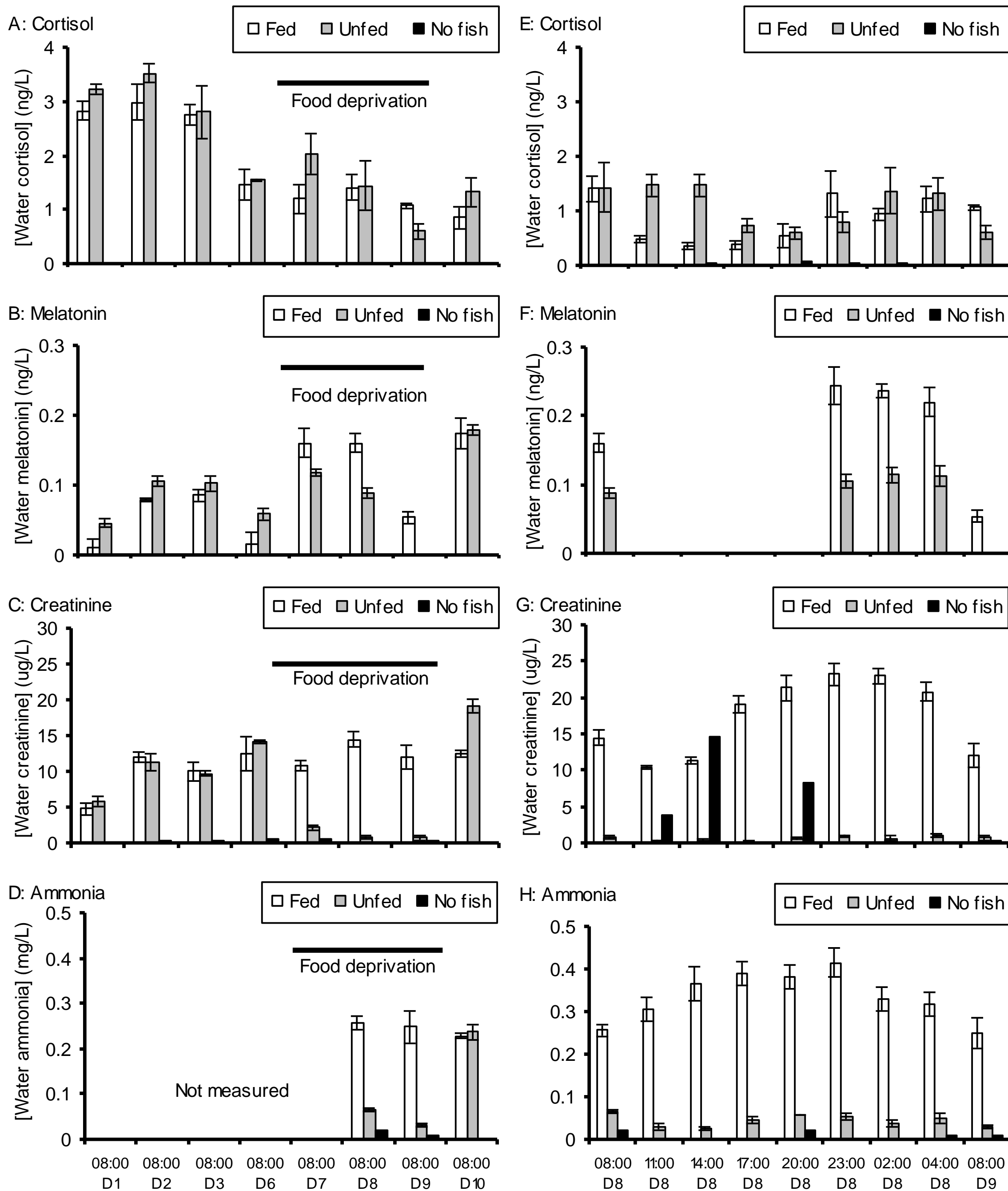

Figure 5: Time series of water cortisol (A), melatonin (B), creatinine (C) and ammonia (D) concentrations at 08:00 on days 1 to 10 (D1-D10) in tanks of rainbow trout exposed to differing feeding treatments (fed, food deprived on days 7-9) and concentrations in a tank receiving feed but containing no fish. Time series of water cortisol (E), melatonin (F), creatinine (G) and ammonia (H) concentrations in tanks of rainbow trout exposed to differing feeding treatments (fed, food deprived) over a 24 h cycle on day 8-9 during food deprivation, and concentrations in a tank receiving feed but containing no fish. Values for tanks with fish are means (±S.E, n=3 tanks per treatment combination).

The results of this experiment show that relatively short term food deprivation can

- cause a cortisol response. The use of the non-invasive cortisol assay enabled sampling at various times of day which illustrated a time-dependence in cortisol response. This finding may go some way to explaining contradictory results in previous studies (see **43**).
- cause a marked decrease in melatonin levels. The change in melatonin observed indicates that short-term food deprivation can have marked effects on physiological processes not immediately associated with either nutrition or stress.

Experiment 4: Effects of temperature and stress (and density) on cortisol, melatonin and creatinine release

This experiment was conducted to examine whether:

- temperature affects cortisol, melatonin and creatinine release rates
- acute stress affects cortisol, melatonin and creatinine release rates

The study was conducted in 12 tanks (30 L) supplied with fresh water at a flow rate of 1.2 L/min/tank. Fish (28 ± 0.5 g; mean ± SEM) were stocked at 20 kg/m^3 at an initial temperature of 10°C. After 3 days the temperature was adjusted at a rate of 1°C/day to provide 6 tanks at each of the treatment temperatures of 7° and 15°C. Eight days after stocking (to minimise the time for differential growth between the temperature treatments), three tanks at each temperature were subject to an acute stress (90 sec aerial emersion). The stress was applied 15 min before the start of the dark period to determine any potential effect of the stress on melatonin release. The experiment was therefore a 2 x 2 treatment design: 2 temperatures (7 and 15°C) and 2 stress treatments (stressed, unstressed control), with 3 replicates of each combination. Following the findings in Experiments 2 and 3 that food deprivation affects melatonin and creatinine release, fish were fed as normal on the day of the stressor application. To examine the impact of the acute stress and temperature on metabolite release rate, water samples were collected 00:45 h and immediately prior to the stress, and 00:45, 01:30, 02:15, 03:00, 04:00, 06:00, 08:00, 12:00 and 36:00 h after the stress.

Cortisol: There was a significant effect of the stress treatment ($p<0.001$) with cortisol release rates increasing over the first few hours in the stress treatments (**Fig 6A**). There was also a significant effect of temperature ($p<0.005$), with a higher cortisol release rate at 15°C than 7°C.

Melatonin: The stress treatment did not affect melatonin release rate ($p>0.2$), although there was a marked effect of temperature ($p<0.0001$) with a higher melatonin release rate at 15°C than 7°C (**Fig 6B**).

Creatinine: The stress treatment did not affect creatinine release rate ($p>0.5$), although again there was a marked effect of temperature ($p<0.0001$) with a higher creatinine release at 15°C than 7°C (**Fig 6C**).

Ammonia: The stress treatment did not affect ammonia release ($p>0.5$), although again there was a marked effect of temperature ($p<0.0001$) with a higher ammonia release at 15°C than 7°C (**Fig 6D**).

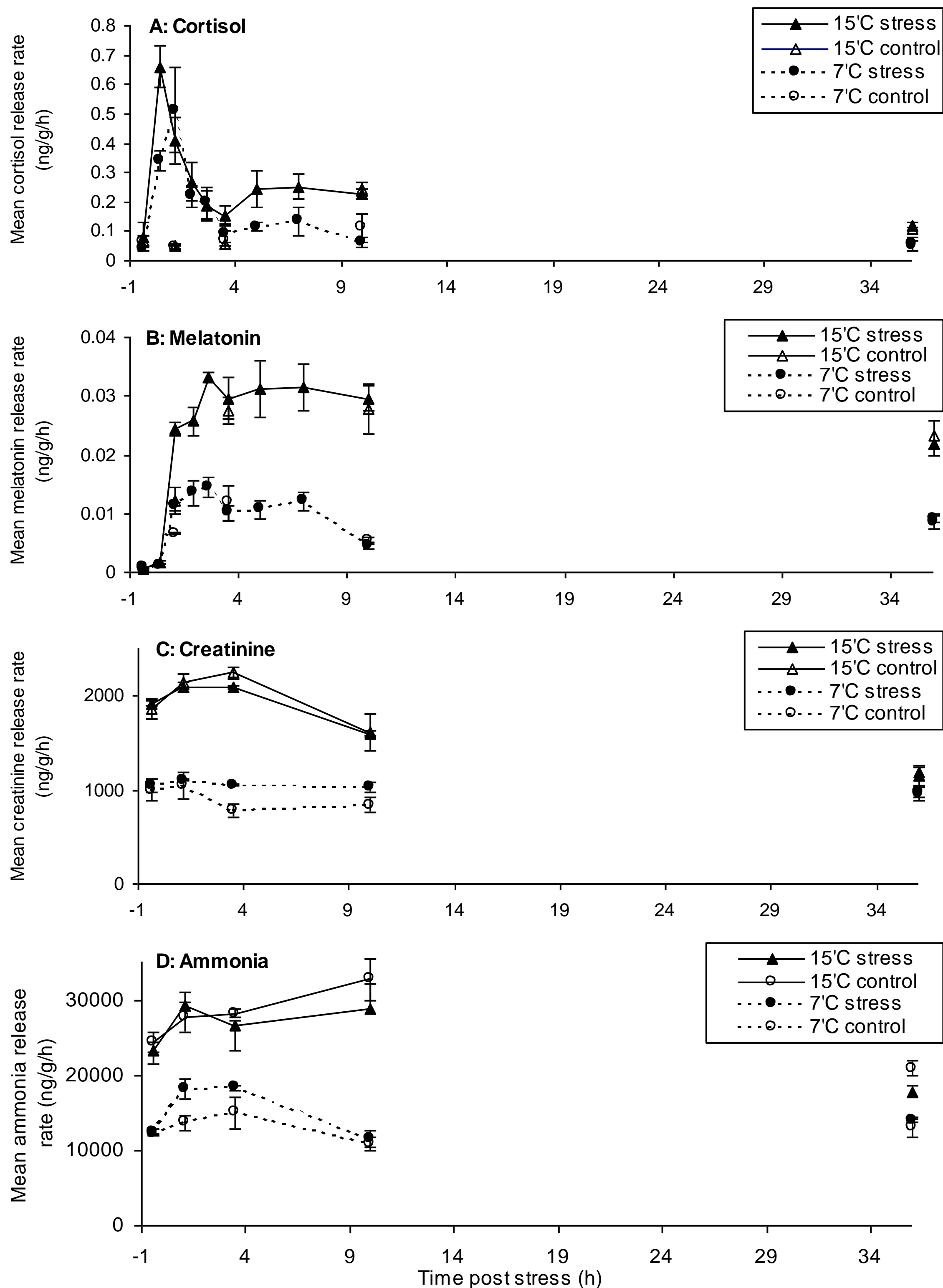

Figure 6: Time series of release rates of cortisol (A), melatonin (B), creatinine (C) and ammonia (D) in tanks of rainbow trout exposed to a handling stress at time =0 or no handling stress at 2 temperatures, 7°C and 15°C. Values for tanks with fish are means (±S.E, n=3 tanks per treatment combination).

Following the results of Experiment 2 indicating an apparent effect of density in the days immediately after stocking, an additional short term (3 day) density study was conducted in conjunction with the above study to examine whether

- density affects metabolite release rates during the acclimation period after stocking

Three additional tanks were stocked at 80 kg/m^3, and these tanks were water sampled just before onset of the light period, at the same time as three of the above 20 kg/m^3 tanks on days 1-3, prior to the temperature changes.

Cortisol: Although cortisol release was apparently higher at 20 kg/m^3 than 80 kg/m^3 on day 1 (**Fig 7A**), it was not significantly affected by density (p=0.14), nor day (p=0.20; interaction with density, p=0.14).

Melatonin: Melatonin release rate was affected by density (p<0.0005) and day (p<0.05; interaction with density, p=0.02). Melatonin release was greater in the 80 kg/m^3 tanks than the 20 kg/m3 tanks on days 2 and 3 (**Fig 7B**).

Creatinine: Creatinine release was similarly affected by density (p=0.0001) and day (p=0.02; interaction with density, p=0.04). Creatinine release rate was higher in the 80 kg/m^3 tanks than the 20 kg/m3 tanks on days 2 and 3 (**Fig 7C**).

Ammonia: Similarly to melatonin and creatinine, ammonia release was affected by density (p=0.003) and day (p=0.03). Ammonia release rate was higher in the 80 kg/m^3 tanks than the 20 kg/m3 tanks (**Fig 7D**).

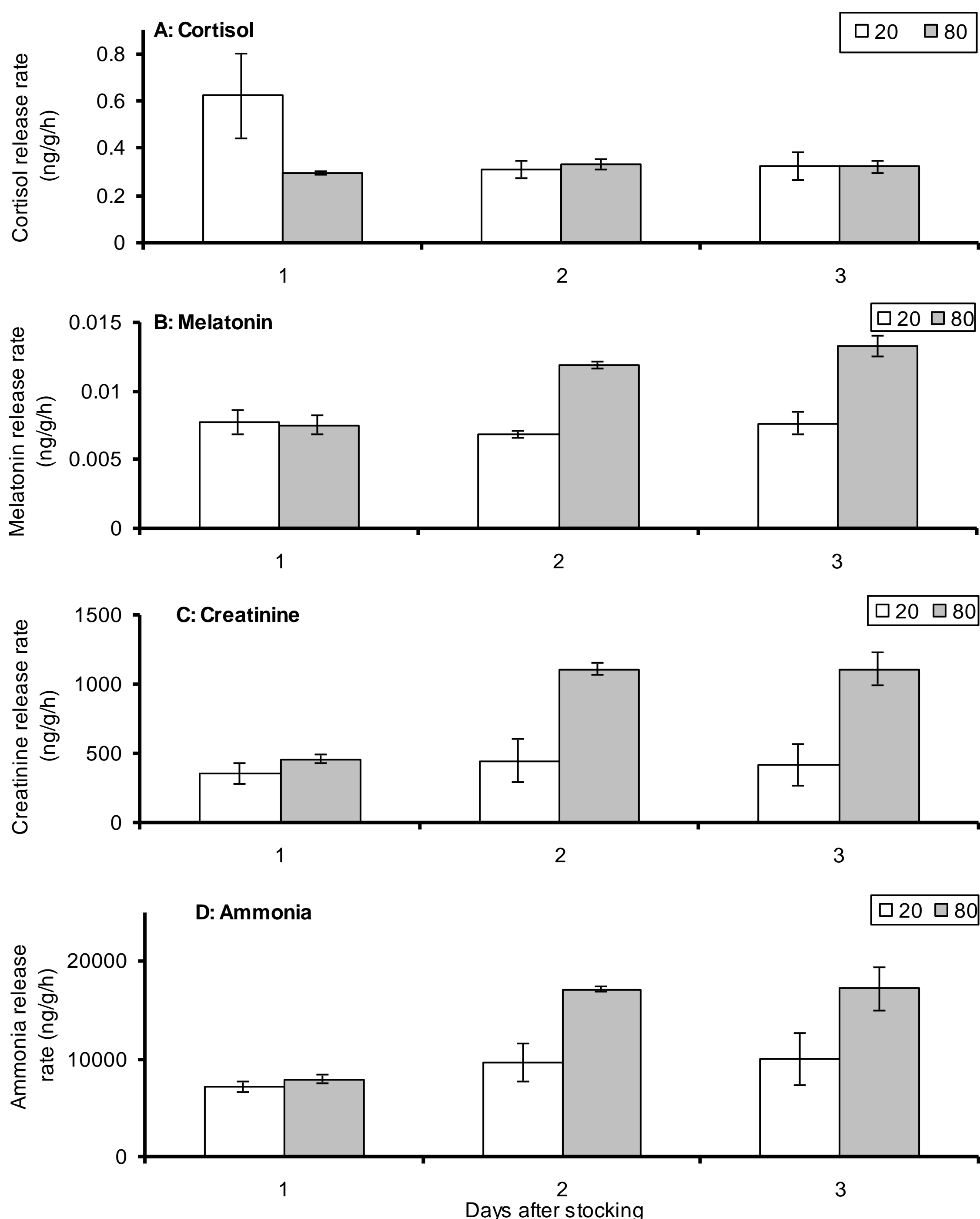

Figure 7: Time series of release rates of cortisol (A), melatonin (B), creatinine (C) and ammonia (D) in tanks of rainbow trout held at a density of either 20 or 80 kg/m^3 for the 3 days after stocking into experimental tanks. Values are means ± S.E, n=3 tanks per treatment.

Normalisers for water cortisol concentration

The primary purpose of Experiments 1 to 4 was to determine whether creatinine and/or melatonin would be suitable normalisers for cortisol. Although melatonin is released into the water via the same route (the gills) as cortisol (**27**), and our experiments indicate that its release rate is unaffected by acute stress and density (after acclimation), it is unsuitable as a normaliser. Melatonin release appears to be affected by feeding rate and adaptation to a change in tank conditions – such factors are likely to accompany stressors and, coupled with the need for night-time sampling, preclude the use of melatonin as a normaliser for cortisol.

Experiment 1 illustrated that creatinine is released into the water via the urine, while cortisol is released via the gills. Ideally a normaliser for cortisol should be released via the same route as cortisol, so that any factors affecting release affect both metabolites equally. Nevertheless, if the normaliser is released at a consistent rate, then it will still be of value. Freshwater fish are viewed as producing large amounts of dilute urine – i.e. they constantly absorb water by osmosis as the solute content of the bodies is much greater than the surrounding water (**44**). This urine, rather than being excreted as a constant "trickle", has been shown to be expelled in pulses (**45**). Creatinine release into the water will therefore be pulsed, in contrast to cortisol release which is thought to be a continuous passive process. Nevertheless, this fact is not thought to present an obstacle to the use of creatinine - as creatinine measurement in the tank water will provide integration over time and over a population.

Creatinine, however, cannot be used as a normaliser in fish as it's release into the water is affected markedly by feeding rate. Experiment 3 illustrated that creatinine release was markedly reduced in fish deprived of food for just 24 hours. Experiment 3 also demonstrated that uneaten food can be a significant source of creatinine in the water. Uneaten food is not believed to be the source of water creatinine in the other experiments – food was not provided (nor regurgitated) in Experiment 1, and the food provided to the fish in Experiments 2, 3 and 4 was recorded by the husbandry technicians as being eaten. It therefore appears that a significant component of the creatinine measured in the water in fish tanks, although released by the fish, is of an exogenous (food) origin, rather than endogenously produced within fish muscle. Consumption of the food by the fish appears to delay the release of the creatinine into the water. Creatinine release is therefore highly affected by feeding rate, will not represent fish muscle mass, and therefore cannot be used as an indicator of fish biomass and a normaliser for cortisol.

Neither of the two candidate normalisers examined within the project – creatinine and melatonin - proved suitable. Other water parameters that are simpler to measure with electronic probes have been suggested to us as indicators of the biomass loading. The amounts of ammonia and carbon dioxide excreted, and the amount of dissolved oxygen taken up, will be labile to the biomass of fish. However, these three water quality parameters will also be affected by fish activity and feeding rate (as illustrated for ammonia in Experiment 3), which would be expected to change with stress. The quest for a normaliser for cortisol therefore continues. It is pertinent to note that in molecular biology, it is now recognised that reference ("housekeeping") genes (analogous to a normaliser as they are assumed to remain constant, and comparisons to other genes reveal up- or down-regulation of target genes) are more variable than previously thought (**46,47**). Just as in molecular biology, we now appreciate that it is very unlikely that we will identify a perfect normaliser that meets all necessary criteria, and selection (from a variety candidates) of the most appropriate for an application, may provide a way forward.

Can something be salvaged from the effort directed to creatinine and melatonin measurement? Our experiments have demonstrated that melatonin levels in fish are more labile to stressors (change in tank conditions, feeding rate, stocking density) than previously envisaged (although see **48-51**), which is of interest given its assumed role in timing gonad development in fish (**52**). Can we suggest that creatinine measurement could be used as an indicator of fish feeding rate? Unfortunately this is not possible as uneaten food also releases creatinine into the water (albeit as a more concentrated pulse). Ammonia measurement provides a more viable option as an indicator of fish feeding rate, due to its simplicity (i.e. not needing a concentration step), the low release from uneaten food, and the potential for real-time monitoring using ammonia electrodes. It is established that salmonids release ammonia at a rate proportional to the amount of feed consumed and its dietary protein content (**53,54**), and routine measurement would represent good practice due to its potential adverse effects on fish health (**44**).

Cortisol responses to disease challenges

Objective 5 relates to examining the potential of using water cortisol concentrations as an early warning of disease within fish populations. Prior to the project we had illustrated that water cortisol surges preceded mortalities in experiments where:

- Seawater Atlantic salmon (*Salmo salar*) were challenged with Infectious Pancreatic Necrosis virus
- Freshwater rainbow trout were challenged with Viral Haemorrhagic Septicaemia virus
- Freshwater Atlantic salmon were challenged with *Aeromonas salmonicida* (the causative agent of the bacterial disease furunculosis)

Within the project, we published these observations within 2 peer-reviewed papers (**34, 55**), together with a review of existing information on cortisol responses to viral, bacterial, fungal and parasitic pathogens. We also

complemented this work by ad-hoc water sampling of various disease challenges undertaken in-house at Cefas Weymouth (conducted for other research projects):

Viral

- Seawater Atlantic salmon challenged with Infectious Pancreatic Necrosis Virus (IPNV)
- Crucian carp (*Carassius carassius*) challenged with Goldfish Haematopoietic Necrosis Virus (GHNV-Cyprinid herpesvirus-2)

Bacterial

- Freshwater rainbow trout challenged with *Renibacterium salmoninarum* (the causative agent of Bacterial Kidney Disease, BKD)
- Freshwater rainbow trout challenged with *Piscirickettsia salmonis* (the causative agent of Salmon Rickettsial Syndrome)

Fungal

- Freshwater salmon challenged with *Saprolegnia parasitica*

Parasitic

- Freshwater salmon and sunbleak (*Leucaspius delineatus*) challenged with *Sphaerothecum destruens* (Rosette agent)

Unknown agent:

- Freshwater rainbow trout challenged with Red Mark Syndrome (RMS possibly caused by the bacterium *Flavobacterium psychrophilum*)

There was an apparent water cortisol response over time in the BKD challenge (**Fig 8**), with cortisol release rate increasing by an order of magnitude over the period when mortality was apparent.

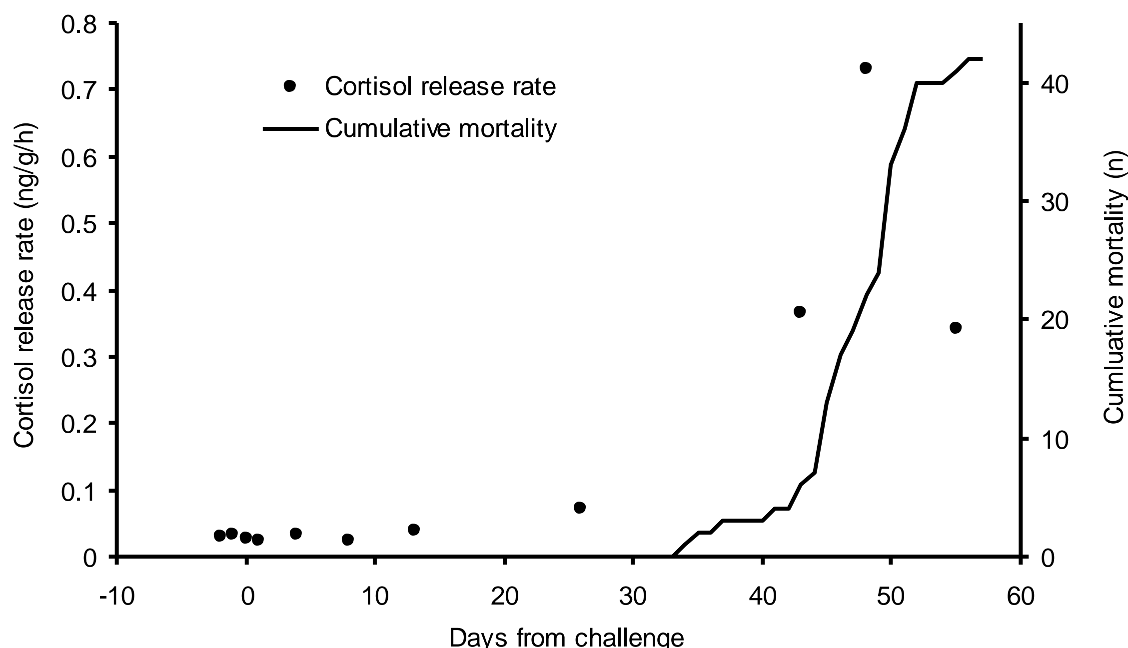

Figure 8: Time series of cortisol release rate and cumulative mortality data from a single tank of freshwater rainbow trout (n=112) challenged with *Renibacterium salmoninarum* (the causative agent of Bacterial Kidney Disease, BKD) on day 0 by i.p. injection.

However, the fact that only a single tank population was maintained for this study prevents statistical analysis. A clear water cortisol signal from infected fish was not apparent in the other challenges sampled which was attributed to a variety of reasons:

- The challenged fish did not succumb to the infection (e.g. GNHV)
- Only a small proportion of the challenged fish population succumbed, with individual mortalities spread over an extended period (e.g. IPNV, *Sphaerothecum*).
- The release of exogenous cortisol (from injected implants to ensure susceptibility to the challenge) obscured any endogenous cortisol effect (*Saprolegnia)*
- The lack of replication prevented clear differentiation between challenged and control tanks (e.g. *Piscirickettsia, RMS)*

We have reviewed the evidence in the literature on cortisol responses to infections in fish (**55**, summarised in **Table 5**). There was a surprising amount of information available - 32 published reports - that had not previously been summarised. Our review highlighted the fact that greatly elevated cortisol levels are a response to a variety of infective agents, but cortisol responses are not a generic feature of infection in fish. It was apparent that a

cortisol response was only associated with those infections in which there was significant tissue damage, moribundity and mortality. This interpretation fits with our own experimental observations of water cortisol surges, or lack thereof.

Table 5: Summary of published studies on cortisol responses to infections in fish. From a total of 32 studies of different pathogen types, the numbers refer to those recording a cortisol increase to infection, and those in which no cortisol response was evident.

Infection	Cortisol response	No cortisol response
Viral	1	1
Bacterial	9	1
Fungal	4	1
Parasitic	8	7

Discussion
Cortisol as an indicator of fish welfare
The tank experiments 2-4 add to the significant existing evidence that cortisol is a valuable indicator of general fish welfare. Not only is cortisol labile to acute handling stress, it also responds to sub-acute changes in housing conditions, density (Expts 2-4) and food deprivation (Expt 3). Such sub-acute cortisol responses were accompanied by changes in other physiological measures: it is unknown whether these other physiological changes were a direct effect of elevated cortisol levels, but the results indicate that, if cortisol levels are elevated, then a wider range of other physiological measures are also disturbed.

The cortisol response after transfer from the stock to experimental tanks lasts several days, longer than the recovery time from just the handling. This illustrates that the change in tank conditions (but not water chemistry) is itself a stressor, and one that is affected by density – at the higher density the fish acclimated faster. This density effect was observed in two independent experiments within the current project and has been observed in previous tank experiments (**28,29**). Possible explanations include:

- more severe behavioural interactions at lower densities during the re-establishment of dominance hierarchies (**56**).
- at higher densities the familiar smell of co-habitants would be stronger - salmonids can recognise and respond to the smell of kin and previous co-habitants (**57,58**).

Again further research is needed to explore these possibilities. Nevertheless, the finding illustrates that the impact of density on welfare is more complicated than the anthropomorphic assumption of a need for space.

At this stage it would be flippant to state that a cortisol stress response in fish does illustrate the presence of negative feelings, but the possibility cannot be dismissed. It is interesting to note that during food deprivation, cortisol levels were only elevated at times of day when food would have been provided: possible explanations include a psychological stress at times when food is not provided when expected (due to daily rhythms or conditioning to the stimuli arising from neighbouring tanks being fed). Further studies would be required to examine this. The disease challenge work illustrates that strong cortisol stress responses only occur in fish during severe infections. Again, it would be flippant to suggest that such cortisol responses indicate that fish are “feeling sick”, although again the possibility of awareness of the escalating gravity of the infection cannot be dismissed. The possible causes and effects of the cortisol response during infection are discussed in **55**.

Water cortisol as an indicator of fish welfare
The extensive sampling done within the WEALTH project also provides further validation of the non-invasive methodology – cortisol responses (or lack of) observed from water samples typically reflected results obtained from invasive blood sampling. Just as basal plasma cortisol levels in salmonids are typically < 10 ng/mL (**59**), we now have a body of data from a variety of experiments showing that basal cortisol release rates in salmonids are typically < 0.1 ng/g/h. The non-invasive method, although integrating over the population and time, is sufficiently sensitive to indicate the presence of acute (handling, confinement) and sub-acute stress (acclimation) and additional treatment (stocking density) effects. The value of the non-invasive assay for obtaining time-series of observations is illustrated by the food deprivation study. The repeated sampling possible with the non-invasive method illustrated that a cortisol response to food deprivation was dependent upon time of day - which may explain the existing equivocal results in the literature (see **43**).

The lack of an identified normaliser for cortisol limits the application of the methodology in its current form (sample collection, extraction, radioimmunoassay, conversion of cortisol concentration into cortisol release rate) to situations where it is known what contact a water sample has had with fish. Nevertheless, a normaliser would not be required if a series of measurements could be taken, as the values would provide a relative measure of stress over time. The development of a real-time cortisol “dipstick” would fulfil this role.

The demonstration of a strong cortisol response to severe infection that precedes mortality suggests that if a quick farm-site measurement system for cortisol in water were to be available, it could be useful as an early warning system for disease problems in fish. Its equivalent in medicine would be 'taking the temperature of a patient'. Early warning would allow fish farmers to limit damage from the disease (e.g. early diagnosis, early treatment, isolating the fish from others, diverting water supplies, destroying stock). Currently, no such 'dipstick' (either in the form of a colour test or electronic meter) is available for cortisol; development of a dipstick is compounded by the problem of the relatively low amounts of cortisol in water (in comparison to those in the blood). However, the problems are unlikely to be insuperable.

As a note of caution, if and when such a "dipstick" test were to be developed, there would still be problems to be faced in applying it as an early warning of disease:

- as discussed above, the amounts of cortisol that the fish release seem to be dependent upon the pathogenicity of the infection: the test thus may have little or no predictive power for chronic or mild diseases;
- in a highly acute disease outbreak advance warning from the water cortisol signal might be too short to enable remedial action;
- diseases affecting the interrenal cells or gills might reduce or prevent the production of cortisol, or the passage of cortisol from the blood into the water respectively;
- cortisol levels in the water (as applied in the present studies) represent an integration of all members of the population; thus it is not possible to tell whether most individuals are partly affected or a few individuals are highly affected;
- interpretation would require knowledge of exposure to other potential stress factors (e.g. handling, grading, exposure to predators);
- interpretation would also need to take into account the fact that fish biomass, water flow rate and temperature affect cortisol concentrations in water; ways to overcome this problem include simultaneous measurement of a normalising compound or regular sampling for cortisol concentrations (i.e. enabling identification of short-term changes).
- in the present studies no records were made of the behaviour of the fish during the course of the experiments; thus it is possible that a simple observation (e.g. reduction in feeding rate) might yet provide a more straightforward and cheaper early-warning system.

Towards a dip-stick test for fish welfare

Although cortisol is a valuable fish welfare indicator, it's measurement in water samples (and blood) is complex and lengthy. There are several distinct procedures involved in present assay of cortisol in water at Cefas: 1) collection of water sample; 2) pumping of water sample through a filter and solid phase extraction cartridge (SPEC); 3) elution of cortisol from SPEC with an organic solvent; 4) evaporation of solvent; 5) radioimmunoassay of cortisol. Although up to 60 samples can be extracted in a day and 240 samples can be assayed at a time, the whole procedure take 3 days. Such measurement limits its use to a retrospective research indicator, of no value as a real-time fish welfare indicator to fish farmers. One objective of the project was to review available methodologies to determine if a 'dipstick' or electronic probe, that can be placed directly in the tank water and give a rapid reading of cortisol concentration, was a tangible research target.

'Dipstick' technology already exists in the medical field. However, it is all geared towards measurement of concentrations of compounds in the ng/mL range (as found in plasma, urine and saliva). The range of cortisol concentrations typically found in fish tanks is 3 orders of magnitude lower, i.e. in the ng/L range. This is why we have to extract and concentrate the cortisol from the water. Although there has been one paper with the word 'cortisol dipstick' in its title (**60**) and at least two patent applications (**61,62**), there are at present no commercially-available dipstick-type methods for cortisol measurement. Even if these existed, they would be optimised for cortisol concentrations in the ng/ml range found in human body fluids. Work is therefore needed to develop sensitive immunoassay methods or adapt existing dipstick technologies.

An intriguing and potentially suitable immunoassay for cortisol has recently been published (**63**) termed a "homogeneous" assay. This means that it is performed in only one phase, namely, the liquid phase, and thus, does not require separation of phases as its heterogeneous counterparts do. As opposed to heterogeneous assays, the signal generation in a homogeneous assay is a direct result of analyte binding, which allows the multiple washing and incubation steps required in an indirect heterogeneous assay format to be eliminated. Moreover, homogeneous assays are usually fast (3-5 minutes) and amenable to miniaturization and automation. The paper (**63**) describes the development of a homogeneous assay for cortisol using the bioluminescent photoprotein aequorin as a reporter molecule. A cortisol derivative was chemically conjugated to the lysine residues of a genetically modified aequorin in order to prepare an aequorin-cortisol conjugate capable of binding anti-cortisol antibodies. The binding of anti-cortisol antibodies to the aequorin-cortisol conjugate resulted in a linear response reflected in the emission of bioluminescence by aequorin. A competitive binding assay was developed by simultaneously incubating the aequorin-cortisol conjugate, the anti-cortisol antibodies, and the sample containing free cortisol. Dose-response curves were generated relating the intensity of the bioluminescence signal with the concentration of free cortisol in the sample. Unfortunately, the sensitivity of the assay is not particularly great (36 ng/L detection limit); water samples would still have to be extracted, but the speed of the assay is attractive.

The **immunoenzymometric assay** is by far the most sensitive steroid assay in the literature. It is based on so-called anti-idiotype antibodies (**64**). In the authors own words: “To overcome the sensitivity limit in immunoassays for small molecules (haptens), we established a noncompetitive immunoenzymometric assay (IEMA) format that can detect attomole-range hapten molecules. We selected 11-deoxycortisol (11-DC; *M*r 346.5), a corticosteroid serving as a diagnostic index for pituitary-adrenal function, as a model target hapten. A fusion of a single-chain Fv fragment (scFv) specific for 11-DC and alkaline phosphatase (ALP) was generated for use as an enzyme-labeled antibody, instead of the conventional chemically linked enzyme-antibody conjugates. After binding reaction of 11-DC and fixed amounts of the fusion protein (scFv-ALP), the unbound fusion protein was removed by incubation with a mouse *â*-type anti-idiotype antibody recognizing the scFv paratope. These complexes were captured by magnetic separation using anti-mouse IgG antibody-coated magnetic beads. Following magnetic sedimentation of the beads, immune complexes of scFv-ALP and 11-DC remained in the supernatant were further purified by capture on microtiter plates with immobilized r-type antiidiotype antibody. As measured fluorometrically, ALP activity from bound immune complexes on the plates increased with increasing 11-DC, which is characteristic of a noncompetitive relationship. This IEMA afforded an extremely low detection limit (20 amol/assay), a very wide measurable range, and practical specificity.” The high sensitivity of this assay, which would be applicable to measurement of cortisol in unextracted water samples, comes at the cost of complexity. At least three reagents would need to be developed specifically for the measurement of cortisol, and the development would not be simple.

Biosensor technology offers several potential advantages over other conventional analytical techniques, including increased assay speed, automation, capability for multi-target analyses, reduced costs of diagnostic testing and portable screening which allows for decentralization from the laboratory environment. Electrochemical devices have traditionally received the major share of the attention in biosensor development. Thick film technology using screen-printing procedure is a widely used method for the simple and fast mass-production of inexpensive disposable electrochemical sensors (50 cents to 1 Euro per sample). This technique consists of depositing inks of different electrochemical properties on an inert substrate in a film of controlled patter and thickness. By using disposable electrodes, it is possible to avoid the time-consuming reconditioning that is required with non-disposable sensors.

Cefas has been in contact with a scientist at the Open University who has been involved in the development of automated biosensor systems for the detection of metabolite levels in veterinary testing, for example oestrus detection by monitoring progesterone in whole milk (**65,66**). The progesterone biosensor is fabricated by depositing monoclonal antibody onto screen-printed carbon electrodes and is operated following the steps of competitive binding between sample and conjugate (alkaline-phosphatase labelled progesterone). The sensor relied upon a reduction in the binding to the sensing surface of labelled metabolite in the presence of endogenous sample metabolite. The 1-napthylphosphate is the enzyme substrate of choice and the 1-naphthol generated in the reaction is electrochemically oxidised upon application of the appropriate potential (300 mV) to the screen printed carbon electrode, producing a signal inversely proportional to the concentration of unlabelled metabolite in milk.

Although this procedure uses an enzyme-labelled steroid as a tracer, the tracer at no time comes into contact with the sample. This means that the chances of the enzyme being damaged by non-steroidal compounds that might be present in the water extracts (a presumed common source of ‘false positive’ matrix effects in Enzyme-linked assays) is very much reduced. This is a very attractive aspect of this procedure. The whole procedure takes about 1 hour, and although this cannot be described as instant, the procedure can be readily automated. The biosensing system detects concentrations of progesterone between 3 and 30 ng/ml in whole fresh milk (normal physiological levels). Although this range is not sensitive enough for measuring cortisol in unextracted water, we nevertheless feel that this would be a good starting point for the development of a rapid sensor. Our thinking goes along these lines:

1) Using already available reagents, modify the existing progesterone biosensor assay for cortisol; use this to replace the present RIA
2) Prepare some high affinity polyclonal antibodies to cortisol and use these to replace the monoclonal antibody; this should give a 10 to 100-fold increase in sensitivity
3) Concurrently, contact the Japanese group for advice and start to develop the reagents required for the ultra-sensitive immunoenzymometric-type assay; use these to replace the more conventional reagents in the biosensor assay – with an expected further 100-fold increase in sensitivity.

An increase in sensitivity of 1000-fold would make the assay easily applicable to unextracted water extracts.

The future: measuring fish metabolites in water to assess health and welfare status?

The assessment of the welfare status of fish is still in its infancy. Direct observation of farmed fish is largely impractical due to their small individual size, large population sizes, the 3 dimensional space occupied, and habitation in water. This project has demonstrated the potential that measuring excreted metabolites holds as a non-invasive means of monitoring the physiological state, health and welfare of fish. Fish farmers are already

alert to the value of measuring oxygen and excretory metabolites (ammonia, carbon dioxide) concentrations as measures of environmental quality; it is a small step to recognise that such or similar measurements can also provide information upon the physiological state of the fish. For example the change in oxygen consumption rate during salmon loading and transport in a well-boat has recently been used to infer stress and recovery (**67**). As discussed above, ammonia monitoring could provide a non-invasive means for monitoring food intake. Even if it proves difficult to develop a rapid test for cortisol in water or the test ultimately proves not to be useful for disease monitoring, the principle itself, of measuring something in the water rather than in the fish, could be extended to other metabolites. Some other compounds excreted into the water by the fish or the pathogens themselves are likely to have predictive power for specific infections.

Although creatinine was ultimately shown to be unsuitable as a normaliser, the development of the methodology and its application was valuable: it demonstrated potential methodological (i.e. salt content of water) and interpretational (metabolites deriving from feed rather than the fish themselves) pitfalls of the metabolite measurement approach. We foresee that, with further research, development and novel thinking, the measurement of metabolites in the water will become more commonplace for assessing the health and welfare of farmed fish. Allied to the non-invasive monitoring of metabolite output, could be "eaves-dropping" on the auditory output of acoustically communicating fish species, for example tilapia and cod (**68,69**).

Outputs & Affiliated work

Four peer-review papers have been published to date from work associated with this project:

Ellis T, Bagwell N, Pond M, Baynes S, Scott AP (2007). Acute viral and bacterial infections elevate water cortisol concentrations in fish tanks. *Aquaculture* **272**, 698-706.

Ellis T, James J, Fridell F, Sundh H, Sundell K, Scott AP (2007). Non-invasive measurement of cortisol and melatonin in seawater Atlantic salmon tanks. *Aquaculture* **272**, 707-716.

Scott AP, Ellis T (2007). Measurement of fish steroids in water – a review. *General & Comparative Endocrinology* **153**, 392-400.

Scott AP, Hirschenhauser K, Bender N, Oliveira R, Earley RL, Sebire M, Ellis T, Pavlidis M, Hubbard PC, Huertas M, Canario A (2008). Non-invasive measurement of steroids in fish-holding water: important considerations when applying the procedure to behaviour studies. *Behaviour* **145 Suppl. SI**, 1307-1328.

Further papers will be drafted on 1) measurement of creatinine excretion by rainbow trout and 2) the effects of density and food deprivation on rainbow trout (combined with data gathered in AW1203 &FC0916).

The work has been presented at national and international / meetings:

- Talk: World Aquaculture conference, Florence, May 2006 (Ellis, T., James, J. & Scott, A.P. A non-invasive cortisol assay for seawater Atlantic salmon. World Aquaculture Society Conference 2006, p. 282.)
- Poster: General & Comparative Endocrinology Conference, Manchester, Sept 2006 (Scott, A.P., Sebire, M., Katsiadaki, I & Ellis T. Non-invasive measurement of fish steroids in water)
- Talk: COST 867 1st meeting, Arcachon, Oct 2006. (Scott, A.P. & Ellis, T. Progress towards a 'dipstick' test for fish welfare: http://www.fishwelfare.com/images/pdf/anchachon/wg2ellis.pdf)
- Talk: COST 867, Varese, Italy, May 2007. Ellis: Cortisol and fish welfare
- Talk: Fish Pheromone and Steroids in Water workshop, Portugal, Sept 2007. Scott, Ellis: Progress in the development of a normaliser for cortisol and other compounds released into the water by fish.
- Talk: Defra Fish Welfare Meeting, Reading, November 2007. Ellis: AW1206: Further development of a non-invasive stress assay for fish
- Talk: COST 867, Krakow, Poland, April 2008. Ellis: Cortisol as an indicator of fish feelings
- Talk: VLA Animal Diseases Conference, Surrey, Sept 2009: Ellis: Developing a non-invasive stress assay for fish

In addition to the research reported here, the Defra support for this project has maintained the expertise at Cefas Weymouth in fish welfare and the measurement of steroids in water. This active research has benefited the quality of the responses to Defra requests for advice on 1) farmed fish welfare in relation to the Council of Europe Recommendations and the Organic Aquaculture Regulations, and 2) on steroid release into the environment from fish farms. Advice to the Home Office ASPA committee on the welfare of experimental fish has also been provided. Furthermore, the leading role that Cefas has played, with Defra's support, in promoting non-invasive steroid measurement is reflected in the uptake of the methodology by the wider scientific community (**31**). Defra's continued support of this work area has also facilitated Cefas involvement in associated fish welfare work:

- University of Edinburgh: Plasma cortisol samples were processed from experiments involving differing handling methods

  Brydges NM, Boulcott P, Ellis T, Braithwaite VA (2009). Quantifying stress responses induced by different handling methods in three species of fish. *Applied Animal Behaviour Science* **116**: 295–301.
- University of Crete: Measurement of cortisol in sea-bass water samples

  Fanouraki E, Papandroulakis N, Ellis T, Mylonas CC, Scott AP, Pavlidis M (2008). Water cortisol is a reliable indicator of stress in European sea bass, *Dicentrarchus labrax*. *Behaviour* **145 Suppl. SI**, 1267-1281.

- University of Oslo: Non-invasive cortisol samples were processed to support work on selective breeding for cortisol responsiveness (associated with behavioural phenotype)
  Kittilsen S, Ellis T, Schjolden J, Braastad BO, Øverli Ø (In press). Determining stress responsiveness in family groups of Atlantic salmon (*Salmo salar*)using non-invasive measures. *Aquaculture*
- EU WEALTH project: water cortisol samples were processed from salmon and sea-bass experiments and contributions were made at meetings and to reports:
  Sundh, H, Olsen RE, Ellis T, Kvamme BO, Fridell F, Taranger GL, Sundell K (In draft). Chronic exposure to low oxygen levels detrimentally affects the intestine of Atlantic salmon (*Salmo salar* L.) postsmolts.
- EU COST Action 867 Wellfish: Welfare of Fish in European Aquaculture: Tim Ellis is the leader of a working group on cortisol
  Ellis T, Yavuzcan, H, Keskin E, López-Olmeda JF, Ruane N (In prep) Cortisol and finfish welfare.
- Cefas: Water cortisol assessment of tagging stress in salmon smolts
  Lower N, Riley WD, Ellis T, Moore A (In prep). The effect of handling and tagging on stress levels in Atlantic salmon smolts (Salmo salar L.) before and after seawater transfer.
- Cefas: Examination of association between cortisol responsiveness of rainbow trout strains and susceptibility to viral infection
  Paley R, Baynes SM, Bagwell N, Ellis T, Pottinger FG (In prep) VHS susceptibility of rainbow trout is associated with cortisol responsiveness.
- Ruskin Anglian University: Non-invasive cortisol samples were processed from a tropical ornamental fish welfare study
- Viking Aquaculture: Water cortisol samples were processed to support work on handling & transport stress in juvenile cod and turbot

Data generated within AW1206 illustrating that human skin contact can be a significant source of steroid contamination of fish tank water samples also lead to a Cefas Seedcorn project (and patent application) examining the potential for non-invasive monitoring of mammalian steroid levels via skin washes. Work within the Seedcorn project also showed that fish release serotonin into the water that can be measured using similar methods to those used for creatinine: the non-invasive measurement of serotonin may provide an indicator of a positive welfare state (as opposed to measurement of cortisol being an indicator of negative welfare state) although much additional background work would be required.

References

1. Ellis T**,** Oidtmann B, St-Hilaire S, Turnbull J, North B, MacIntyre C, Nikolaidis J, Hoyle I, Kestin S, Knowles T (2008). Fin erosion in farmed fish. P121-149 in *Fish Welfare*, Branson E. (Ed), Blackwell, Oxford.
2. Anon. (1996). *FAWC Report on the Welfare of Farmed Fish*. Farm Animal Welfare Council, Surbiton, 51p.
3. Anon (2009). Scientific opinion of the panel on animal health and welfare on a request from European Commission on general approach to fish welfare and to the concept of sentience in fish. *The EFSA Journal* **954**, 1-26.
4. Dawkins MS (2006). A user's guide to animal welfare science. *Trends in Ecology and Evolution* **21**, 77-82.
5. Somerville BA, Broom DM (1998). Olfactory awareness. *Applied Animal Behaviour Science* **57**, 269-286.
6. Chandroo KP, Duncan IJH, Moccia RD (2004). Can fish suffer?: perspectives on sentience, pain, fear and stress. . *Applied Animal Behaviour Science* **86**, 225-250.
7. http:\\www.fawc.org.uk/freedoms.htm
8. Yeates JW, Main DSCJ (2008). Assessment of positive welfare: a review. *Veterinary Journal* **175**, 293-300.
9. Huntingford FA, Adams C, Braithwaite VA, Kadri S, Pottinger TG, Sandöe P, Turnbull JF (2006). Review Paper: Current issues in fish welfare. *J Fish Biol* **68**, 332-372.
10. Arlinghaus R, Cooke SJ, Schwab A, Cowx IG (2006). Fish welfare: a challenge to the feelings-based approach, with implications for recreational fishing. *Fish & Fisheries* **8**, 57-71.
11. www/bbsrc.ac.uk/organisation/policies/public_interest/animal_welfare.pdf
12. Korte, S.M. (2001). Corticosteroids in relation to fear, anxiety and psycopathology. Neuroscience & Biobehavioral Reviews 25, 117-142.
13. Levine A, Zagoory-Sharon O, Feldman R, Lewis JG, Weller A (2007). Measuring cortisol in human psychobiological studies. *Physiology & Behaviour* **90**, 43-53.
14. Mazeaud MM, Mazeaud F (1981). Adrenergic response to stress in fish. In *Stress and Fish* (Pickering, A. D., ed.), pp. 49–75. London: Academic Press.
15. Donaldson EM (1981). The pituitary-interrenal axis as an indicator of stress in fish. In: Pickering, A.D. (Ed.), *Stress and Fish*. Academic Press, London, pp. 11-47.
16. Barton BA (2002). Stress in fishes: a diversity of response with particular reference to changes in circulating corticosteroids. *Integrative & Comparative Biology* **42**, 517-525.
17. Anderson DP (1990). Immunological Indicators: effects of environmental stress on immune protection and disease outbreaks. *Am Fish Soc Symp* **8**, 38-50.
18. Rose JD (2002). The neurobehavioural nature of fishes and the question of awareness and pain. R*ev Fish Sci* **10**, 1-38.
19. Rose JD (2007). Athropomorphism and 'mental welfare' of fishes. *Diseases of Aquatic Organisms* **75**, 139-154.

20. Ellis T, Yavuzcan, H, Keskin E, López-Olmeda JF, Ruane N (In prep) Cortisol and finfish welfare.
21. Turnbull J, Bell A, Adams C, Bron J, Huntingford F (2005). Stocking density and welfare of cage farmed Atlantic salmon: application of a multivariate analysis. *Aquaculture* **243**, 121-132.
22. North BP, Turnbull JF, Ellis T, Porter MJ, Migaud H, Bron J, Bromage NR (2006). The impact of stocking density on the welfare of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **255**, 466-479.
23. Varsamos S, Flik G, Pepin JF, Wendelaar Bonga SE, Breuil G (2006). Husbandry stress during early life stages affects the stress response and health status of juvenile sea bass, *Dicentrarchus labrax*. *Fish Shellfish Immunol* **20**, 83-96.
24. Lane J (2006). Can non-invasive glucocorticoid measures be used a reliable indicators of stress in animals. *Animal Welfare* **15**, 331-342.
25. Ellis T, James JD, Stewart C, Scott AP (2004). A non-invasive stress assay based upon measurement of free cortisol released into the water by rainbow trout. *Journal of Fish Biology* **65**, 1233-1252.
26. Lower N, Moore A, Scott AP, Ellis T, James JD, Low PJ (2005). A non-invasive method to assess the impact of electronic tag insertion on stress levels in fish. *Journal of Fish Biology* **67**, 1202-1212.
27. Ellis T, James JD, Scott AP (2005). Branchial release of free cortisol and melatonin by rainbow trout. *J Fish Biol* **67**, 535-540.
28. http://randd.defra.gov.uk/Document.aspx?Document=AW1203_6977_FRP.doc
29. http://randd.defra.gov.uk/Document.aspx?Document=FC0916_2950_FRP.doc
30. Scott AP, Ellis T (2007). Measurement of fish steroids in water – a review. *General & Comparative Endocrinology* **153**, 392-400.
31. Scott AP, Hirschenhauser K, Bender N, Oliveira R, Earley RL, Sebire M, Ellis T, Pavlidis M, Hubbard PC, Huertas M, Canario A (2008). Non-invasive measurement of steroids in fish-holding water: important considerations when applying the procedure to behaviour studies. *Behaviour* **145 Suppl. SI**, 1307-1328.
32. Mason GJ, Cooper JJ, Clarebrough C (2001). Frustrations of fur-farmed mink. *Nature* **410**, 35-36.
33. James JD, Scott AP, Ellis T (2004). Water based measurement of rainbow trout melatonin. *Journal of Fish Biology* **65**, 1298-1304.
34. Ellis T, James J, Fridell F, Sundh H, Sundell K, Scott AP (2007) Non-invasive measurement of cortisol and melatonin in seawater Atlantic salmon tanks. *Aquaculture* **272**, 707-716.
35. Wyss M, Kaddurah-Daouk R (2000). Creatine and creatinine metabolism. Physiological Reviews 80, 1007-1211.
36. Hickman CP Jr, Trump BF (1969). The kidney In Fish Physiology Volume I Excretion, Ionic Regulation, and metabolism (Hoar, WS, Randall DJ eds), pp 91-239. London: Academic Press.
37. Foster RP, Goldstein L (1969). Formation of excretory products. In Fish Physiology Volume I Excretion, Ionic Regulation, and metabolism (Hoar, WS, Randall DJ eds), pp 313-350. London: Academic Press.
38. Holloway HL, Shoemaker CA (1993). Humoral creatinine in walleye (*Stizostedion vitreum*). *Great Basin Naturalist* **53**, 199-202.
39. Kajimura M, Croke SJ, Glover CN, Wood CM (2004). Dogmas and controversies in the handling of nitrogenous wastes: the effects of feeding and fasting on the excretion of ammonia, urea and other nitrogenous waste products in rainbow trout. *Journal of Experimental Biology* **207**, 1993-2002.
40. http://chemistry.uca.edu/Projects/Quant98/creatinine-iex-98.pdf
41. Anon (2003). Oasis® sample extraction products. Waters Corporation.
42. Tytler P, Hawkins AD (1981). Vivisection, anaesthetics and minor surgery. In Aquarium Systems (Hawkins, AD, ed.), pp. 247-278. London: Academic Press.
43. Pottinger TG, Carrick TR, Yeoman WE (2002). The three-spined stickleback as an environmental sentinel: effects of stressors on whole-body physiological indices. *J Fish Biol* **61**, 207-229.
44. Wedemeyer GA (1996). Physiology of fish in intensive culture systems. Chapman & Hall, New York, 232p.
45. Appelt CW, Sorensen PW (2007). Female goldfish signal spawning readiness by altering when and where they release a urinary pheromone. *Animal Behaviour* **74**, 1329-1338.
46. Frost P, Nilsen F (2003). Validation of reference genes for transcription profiling in the salmon louse, *Lepeophtheirus salmonis*, by quantitative real-time PCR. *Veterinary Parasitology* **118**, 169-174.
47. Jorgensen SM, Kleveland EJ, Grimholt U, Gjoen T (2006). Validation of reference genes for real-time polymerase chain reaction studies in Atlantic salmon. *Marine Biotechnology* **8**, 398-408.
48. Kulczykowska E (2001). Responses of circulating arginine vasotocin, isotocin, and melatonin to osmotic and disturbance stress in rainbow trout (Oncorhynchus mykiss). *Fish Physiology & Biochemistry* **24**, 201-206.
49. Larson ET, Winberg S, Mayer I, Lepage O, Summers CH, Øverli Ø (2004). Social stress affects circulating melatonin levels in rainbow trout. *Gen Comp Endocrinol* **136**, 322-327.
50. Ceinos RM, Polakof S, Illamola AR, Soengas JL, Míguez JM (2008). Food deprivation and refeeding effects on pineal indoles metabolism and melatonin synthesis in the rainbow trout *Oncorhynchus mykiss*. *Gen Comp Endocrinol* **156**, 410-417.
51. Mancera JM, Vargas-Chacoff L, García-Ló A, Kleszczyńska A, Kalamarz H, Martínez-Rodríguez G, Kulczykowska E (2008). High density and food deprivation affect arginine vasotocin, isotocin and melatonin in gilthead sea bream (Sparus auratus). Comp Biochem Physiol A 149, 92-97.
52. Bromage N, Porter M,. Randall C (2001). The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. Aquaculture 197, 63-98.
53. Wedemeyer GA (1996). *Physiology of fish in intensive culture systems*. New York: Chapman & Hall. 258pp.

54. Wagner E., Miller SA, Bosakowski T (1995). Ammonia excretion by rainbow trout over a 24-hour period at two densities during oxygen injection. *Progressive Fish-Culturist* **57**, 199-205
55. Ellis T, Bagwell N, Pond M, Baynes S, Scott AP (2007). Acute viral and bacterial infections elevate water cortisol concentrations. *Aquaculture* **272**, 698-706.
56. Ellis T, North B, Scott AP, Bromage NR, Porter M, Gadd D (2002). The relationships between stocking density and the welfare of farmed rainbow trout. *J Fish Biol* **61**, 493-531.
57. Brown GE, Brown JA (1992). Do rainbow trout and Atlantic salmon discriminate kin? *Canadian Journal of Zoology* **70**,1636-1640.
58. Hirvonen H, Ranta E, Piironen J, Laurila A, Peuhkuri N (2000). Behavioural responses of naive Arctic charr young to chemical cues from salmonid and non-salmonid fish. *Oikos* **88**, 191-199.
59. Sumpter, JP (1997). The endocrinology of stress. p 95-118 in Iwama, GK, Pickering, AD, Sumpter, JP, Schreck, CB (eds) *Fish stress and health in aquaculture*, Cambridge University Press.
60. Leung W, Chan P, Bosgoed F, Lehmann K, Renneberg I, Lehmann M, Renneberg R (2003). One-step quantitative cortisol dipstick with proportional reading. *Journal of Immunological Methods* **281**, 109-118.
61. Hutchinson TD, Swaniker HP (1999). Wound diagnosis by quantitating cortisol in wound fluids. In United States Patent. United States: Johnson & Johnson Medical, Inc. (Arlington, TX).
62. Lawrence DS, Sample JL (2004). Cortisol sensor. In United States Patent: The John Hopkins University (Baltimore, MD).
63. Rowe L, Deo S, Sofner J, Ensor M, Daunert S (2007). Aequorin-based homogeneous cortisol immunoassay for analysis of saliva samples. *Bioconjugate Chemistry* **18**, 1772-1777.
64. Kobayashi N, Iwakami K, Kotoshiba S, Niwa T, Kato Y, Mano N, Goto J (2006). Immunoenzymometric assay for a small molecule,11-deoxycortisol, with attomole-range sensitivity employing scFv-enzyme fusion protein and anti-idiotype antibodies. *Analytical Chemistry* **78**, 2244-2253.
65. Pemberton R, Hart J, Mottram TT (2001). An electrochemical immunosensor for milk progesterone using a continuous flow system. *Biosensors and Bioelectronics* **16**, 715-723.
66. Velasco-Garcia M, Mottram T (2001). Biosensors in the livestock industry: an automated ovulation prediction system for dairy cows. *Trends in Biotechnology* **19**, 433-434.
67. Farrell, APT (2006). Bulk oxygen uptake measured with over 60,000 kg of adult salmon during live-haul transportation at sea. *Aquaculture* **254,** 646-652.
68. Longrie N, Fine ML, Parmentier E (2008). Innate sound production in the cichlid *Oreochromis niloticus. Journal of Zoology* **275,** 413-417.
69. Finstad JL, Nordeide JT (2004). Acoustic repertoire of spawning cod, *Gadus morhua. Environmental biology of Fishes* **70**, 427-433.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

Please see above - due to the limited capacity for this box to expand, the references are included at the end of the previous section.