

***Investigating Non-invasive Measures of Stress in  
Ornamental Fish***

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## Abstract

The transport of ornamental marine fish may cause stress, which to date has been the subject of limited research. The present study aimed to characterise the behavioural and physiological responses to simulated transport stress in the common clownfish *Amphiprion ocellaris* (Cuvier, 1830) with the additional goal of validating non-invasive measures of water cortisol in a marine teleost for the first time. Behaviour and physiology of the animals was measured at different stages of transport (from initial capture and handling up to 72 hours transport time) and water quality measurements were taken from the transport water at key sampling points. In a second experiment biological filtration materials (“Bioballs” with denitrifying bacteria) were added to the transport bag to determine if stress was reduced when water quality was improved. The results of the study suggest that capture, handling and transport are stressful for clownfish, and the stress response appears to peak between 24 and 48 hours after the onset of the stressor. Water-borne cortisol was found to be a valid alternative to invasive methods of sampling, although only an average of 53% cortisol was recovered from sea water. Although handling and confinement appeared to be highly significant factors in eliciting the stress response water quality measurements revealed that fish are temporarily subjected to relatively high concentrations of ammonia as transport time increased, which may contribute to long-term effects on the health of the animals. This was reflected in an increased latency to feed and reduced social behaviours in fish transported for 24 hours or longer. Improving water quality did reduce the concentration of ammonia present; however, fish still exhibited elevated cortisol excretion suggesting that water quality is not the primary stressor associated with transport. Thus, the duration of transport should be restricted to a maximum of 24 hours to reduce the stress associated with this practice.

A separate study investigated the potential for beauty treatment ‘fish spas’ to elicit stress in the freshwater cyprinid fish *Garra rufa* (Heckel, 1843). Water cortisol was measured non-invasively to determine if stress was reduced through the provision of environmental enrichment / furnishings, and whether stocking density influenced stress. Water quality was monitored to determine the effects of stocking density on environmental parameters (pH, ammonia, nitrite and nitrate). Finally, the effect of

human hands being placed into the aquarium was investigated, to determine whether this influenced stress.

Three hundred *G. rufa* were used in total, with groups being allocated randomly to one of four treatment groups: OP/B (optimum stocking density / barren tank – i.e. no enrichment); OP/E (optimum stocking density / enriched tank); OS/B (overstocked / barren tank); and OS/E (overstocked / enriched tank). Human hands were placed in each tank, and water samples were collected before and after for measurement of cortisol by ELISA, and to determine water quality parameters.

The results revealed that overstocking tanks with *G. rufa* produced relatively higher baseline cortisol levels, suggesting that stocking density may have a significant effect on stress levels. The addition of furnishings into the aquarium did not reduce baseline cortisol levels in the fish prior to the introduction of human hands. However, fish maintained under enriched conditions exhibited a greater cortisol response when compared to individuals in barren tanks. It is hypothesised that the provision of enrichment reduces the available space for fish following the introduction of human hands, thereby increasing stress. Further studies are required to attempt to determine the effect of enrichment based upon the results of the present study. Feeding on human hands resulted in an elevated cortisol response from three out of the four treatment groups (with the exception of OP/B), with the results suggesting that either 1) the lower (i.e. optimal) stocking density and lack of enrichment in holding tanks is preferable for *G. rufa* fish welfare, or 2) the elevated cortisol reflected a response to a rewarding stimulus and is linked to increased foraging.

Overall, the results of these studies have shown that water cortisol measurements are a valid means of assessing physiological stress in two species of fish in different contexts. This negates the need for invasive sampling and is an important refinement to existing protocols where fish are killed for plasma or whole body samples. The results also highlight the welfare impacts of transport and overstocking of ornamental fish providing valuable evidence that may be relevant to improving the husbandry and guidelines with respect to the ornamental fish industry.

# Contents

<b>Abstract</b>	i
<b>Contents</b>	iii
<b>List of Tables</b>	v
<b>List of Figures</b>	vi
<b>Acknowledgements</b>	viii
 <b>Chapter One: General Introduction</b>	 <b>1</b>
Definitions of animal welfare	1
The Five Freedoms	4
The vertebrate stress response	5
The teleost stress response	8
Measurement of stress in fish	10
Issues affecting fish welfare	19
Conclusions	28
 <b>Chapter Two: The Welfare of Ornamental Marine Fish during Transport</b>	 <b>29</b>
Introduction	29
Methods	31
Subjects and husbandry	31
Experiment 1: The impact of transport duration on water quality, cortisol excretion and behaviour	33
Experiment 2: Does improving water quality reduce stress in clownfish during transport?	38
Statistical Analysis	38
Results	39
Experiment 1	39
Experiment 2	45
Discussion	49
Conclusions	54
 <b>Chapter Three: The Impact of Stocking Density and Environmental Enrichment on the Stress Response in <i>Garra rufa</i> used in Beauty Treatments</b>	 <b>55</b>
Introduction	55
Potential welfare issues associated with ‘fish spas’	56
Methods	59
Animal husbandry	59
Experimental design	59
Statistical Analysis	63
Results	63
Discussion	67
Conclusions	70

<b>Chapter Four: General Discussion</b>	<b>72</b>
Transport of ornamental fish	72
The use of <i>Garra rufa</i> in fish spas	74
Improvements in fish welfare	75
Conclusion	77
<b>References</b>	<b>78</b>
<b>Appendices</b>	
Appendix 1: Ethogram for <i>Amphiprion ocellaris</i>	92
Appendix 2: Table showing recovery of known cortisol standards from SPE C18 (Sep-Pak®) cartridges	94
Appendix 3: The relationship between body weight and water cortisol excretion in common clownfish	95
Appendix 4: Protocol for Sep-Pak® water extractions	96
Appendix 5: Cortisol radioimmunoassay (RIA) procedure	98

## List of Tables

### Chapter Two

2.1	The number of fish that fed over two hourly intervals after simulated transport at 0 (30 min acclimation only), 2, 12, 24, 48 and 72 h over the 6 h observation period ( $P < 0.001$ ; $N = 36$ ).	44
2.1	The number of fish that fed over two hourly intervals after simulated transport at 24, 48 and 72 h over the 6 h observation period in the presence of Bioballs ( $P < 0.25$ ; $N = 36$ ).	49

### Chapter Three

3.1	Treatment groups showing stocking density and provision of enrichment using <i>Garra rufa</i> fish ( $n = 50$ individuals; $n = 6$ groups per treatment).	60
3.2	Mean cortisol concentration with standard error before and after presentation of hands in tanks containing <i>Garra rufa</i> fish held under optimally stocked (OP) or overstocked (OS) density and under barren (B) or enriched (E) conditions ( $n = 6$ tank per treatment). T test analysis is also shown with significant P values in bold.	65

## List of Figures

### Chapter One

1.1	Summary of the vertebrate stress response (SNS = sympathetic nervous system).	7
1.2	Summary of the teleost primary, secondary and tertiary stress responses	10
1.3	Fish transport vehicle with GRP holding tanks, each equipped with air diffusers linked to a compressor to ensure adequate oxygenation during transit	22
1.4	Typical packing used for air transport of tropical fish	26
1.5	Spotted puffer ( <i>Tetraodon nigroviridis</i> ) in transport bag following shipment from the Far East to the UK	27
1.6	Captive-farmed silver arowana ( <i>Osteoglossum bicirrhosum</i> ) packed individually for air transport from the Far East in heat-sealed polythene bags	27

### Chapter Two

2.1	The relationship between whole body cortisol (ng/g) and water cortisol (pg/g) in common clownfish ( $R_s=0.987$ ; $P<0.001$ ; $n=20$ )	39
2.2	plots showing median whole body cortisol in common clownfish with increasing simulated transport time from 0 (30 minute acclimation) to 72 hours ( $n = 20$ )	40
2.3	Water cortisol concentration (pg/L) after transport durations of 0 (30 min acclimation = .50), 2, 24, 48 and 72 h in common clownfish ( $N = 36$ )	41
2.4	Total Ammonia Nitrogen (TAN, ppm) measured after differing transport durations from 0 (30 min acclimation = .50) up to 72 h ( $N = 36$ )	42
2.5	The relationship between water cortisol concentrations (pg/L) and total ammonia nitrogen (ppm) ( $N = 33$ )	43
2.6	Median frequency ( $\pm$ IQR) of social behaviour performed by common clownfish after simulated transport of 0, 2, 24, 48 and 72 h ( $N = 6$ per group). Each bar represents the mean $\pm$ SEM, $n = 6$	44

2.7	Boxplots showing the water cortisol concentration in transported clownfish from 24 to 72h duration when no filter material was present (Control) and when Bioballs filter material was present (N = 51)	45
2.8	Boxplots showing a significant difference between water cortisol measurements after 48 h transport in clownfish with no filter material (Control) and those transported with Bioballs (N = 18)	46
2.9	Boxplots exhibiting water cortisol concentrations from clownfish transported for 24, 48 or 72 h with no filter media (Control) or Bioballs (BB; N = 51)	47
2.10	Boxplots showing performance of social behaviour in clownfish after simulated transport for 24, 48 and 72 h in the presence of Bioballs (N = 26).	48



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## **Chapter 1: General Introduction**

### **Definitions of animal welfare**

According to Volpato, Gonçalves-de-Freitas and Fernandes-de-Castilho (2007), welfare can be defined as a state in which an animal ‘feels well’, and the authors question the validity of phrases such as ‘poor welfare’, due to the apparent oxymoronic nature of such terms. Semantics aside, various definitions of ‘welfare’ have been proposed, although the subject is a highly complex one, with no clear consensus on how welfare should actually be defined or measured. Fish present interesting challenges for the measurement of welfare due to the differences in their behaviour and physiology compared with terrestrial mammals. Nevertheless, fish welfare is an area which can be investigated scientifically, in spite of the difficulties in defining and measuring welfare.

Several approaches have been proposed to measure the welfare of animals, although three main methodologies have been extensively developed. Specifically, these definitions are feelings based, function based or use comparison with the natural lives of wild animals. These have varying emphases along a continuum from philosophical arguments to those concerned purely with empirically-measurable biological factors, with some authors adopting a synthesis of more than one approach (Dawkins, 2012).

#### *Feelings-based*

Definitions employing the subjective experiences of the animal are generally termed ‘feelings-based’. The premise underlying feelings-based definitions is that the animal should ‘feel well’ for good welfare to apply. However, this also means the individual can experience adverse welfare states such as pain, fear and suffering (Fraser, 2008). Assessing the internal subjective state of any animal is obviously very difficult and the relevance of the feelings-based approach to assessing welfare has been debated. Nonetheless some experimental approaches have been attempted, such as self-medication of analgesic-laced food when animals are in pain. For example, rats have

been shown to self-medicate on buprenorphine contained in gelatin following surgery (Flecknell, Roughan & Stewart, 1999). Furthermore, it has been demonstrated that rats will self-medicate intravenous morphine when suffering from artificially-induced arthritis (Lyness *et al.*, 1989). Determining whether the animal will pay a cost to accessing a valuable resource is one way of determining the value of that resource to the animal, and may indicate the extent to which welfare may be compromised if it is withheld, which may inform opinions about how that animal is 'feeling' (Mason, Cooper & Clareborough, 2001). Behaviour is measured to give an indication of the animal's emotional state; feelings such as fear may be expressed by animals, and have evolved to protect the animal in times of danger (Fendt & Fenselow, 1999). Whilst such approaches may give some insight into how an animal 'feels', there are difficulties with a feelings-based approach. It has been criticised for being highly subjective and open to observer bias or anthropomorphic interpretations, and it is often difficult to interpret behaviours in terms of 'feelings', although the results may suggest lapses in welfare (Dawkins, 1980). There exists a range of opinions on the value of feelings-based definitions of welfare. Some scientists (e.g. McGlone, 1993) argue for instance, that it is fundamentally impossible to 'know' how an animal is feeling, and that feelings have no place in assessment of animal welfare. This viewpoint relies upon the measurement of purely physiological criteria such as growth and reproduction to assess an animal's welfare status. Others, however (e.g. Duncan, 1993; 1996) maintain that feelings-based judgements are at the very heart of any discussion on animal welfare concepts. Feelings-based approaches to fish welfare may be applied, despite the challenges presented. For an insight into the polarised opinions generated during such debate, see Huntingford *et al.*, (2006), Arlinghaus *et al.*, (2007) and Huntingford *et al.*, (2007).

### *Function-based*

Function-based definitions of animal welfare measurement concern an animal's ability to cope with its environment. Various aspects can be measured, for example growth, incidence of disease, fecundity and behavioural indicators, as well as physiological factors such as stress hormones. These parameters provide robust, experimental measurements of welfare and as such provide convincing evidence on

the state of an animal. The premise here is that if the animal is in good health, it should grow well, have a healthy immune system and be free of disease, be actively reproducing and have low stress indicators. Thus, biological functioning definitions of welfare provide empirical measurements that welfare assessment can be informed by. For example, a study by Molony and Kent (1997) reveals that lambs undergoing castration exhibit abnormal postures, as well as experiencing an elevated cortisol response, following the procedure. Sneddon, Braithwaite and Gentle (2003) investigated the response of rainbow trout when acetic acid and bee venom were administered to the lips. The fish subjected to analgesics demonstrated a range of atypical behaviours including rocking on the pectoral fins, rubbing of the affected area on the aquarium substrate, cessation of feeding and increased respiratory rate. These abnormal behaviours were reduced by administering morphine, a painkiller (Sneddon, 2003<sub>a</sub>).

However, whilst function-based measurement of welfare may provide objective criteria, it has been argued by, for example, Dawkins (1980) that this may not provide the complete picture. For example, a shoaling fish maintained in isolation may appear to be perfectly healthy and fulfil all function-based criteria for acceptable welfare even though it cannot fulfil its normal behavioural repertoire. Measurement of stress indicators does generally reflect homeostatic challenges but often acute stress responses help the animal to cope with a novel threat. Therefore, high cortisol production may reflect a natural coping mechanism and does not necessarily mean poor welfare. Alternatively, chronic stress results in impaired immune function, reduction in reproductive output and reduced growth potentially leading to mortality. Thus, biological functioning measures do need to be interpreted cautiously in the context they are being measured (Keeling & Jensen, 2002).

### *Natural lives*

The ‘natural lives’ approach to quantifying welfare involves a direct comparison between the observed behaviours of captive animals and their wild counterparts. This has been proposed as a measurement of welfare in, for example, calves in intensive-rearing settings (Wagenaar & Langhout, 2007), with the aim of reducing discrepancies between the behaviours exhibited by calves in such systems with those

maintained in less intensive, more ‘natural’ systems. The premise in natural lives definitions of animal welfare are that if they are in a good welfare state then the animal’s behaviour should be similar to that seen in a natural environment. One should also provide all the resources the animals need to perform their natural behavioural repertoire (or artificial analogues of those resources). Therefore, animals should lead a natural life and should be able to exhibit natural behaviours shown by their wild counterparts or ancestors (Rollin, 1993). However, in captivity the motivation to perform normal feeding behaviour such as foraging may be reduced since food is provided to animals, so caution may be necessary when inferring from the comparison of captive animal behaviour with wild animal behaviour. Conditions in production systems may also remove the motivation to perform some behaviours. For example, adult Atlantic salmon (*Salmo salar*) may migrate many thousands of miles in the oceans searching for food yet farmed Atlantic salmon are held in relatively small sea cages but are provided with plentiful food. Does this remove the motivation (or simply the opportunity) for these fish to migrate, or does this explain why they constantly swim in a circular fashion in the cage?

### **The Five Freedoms**

There is no general consensus on the definition of animal welfare, and often two or three approaches are applied simultaneously. With regard to the management of production animals, clear guidelines have been produced and although these were originally applied to the farm context, they are now universally applied to captive animals in experimentation, public exhibit such as zoos and aquaria, as well as to companion animals. The ‘five freedoms’ have been proposed as a framework in which welfare can be maintained, with each of the five ‘domains’ representing key aspects of an animal’s psychological and physiological needs. The five freedoms were developed by the Farm Animal Welfare Council (FAWC) in 1979 following the Brambell Report of 1965 (Report of the Technical Committee to Enquire into the Welfare of Animals kept under Intensive Livestock Husbandry Systems) (FAWC, 2009). The five freedoms are as follows:

- Freedom from hunger and thirst

- Freedom from discomfort
- Freedom from pain, injury or disease
- Freedom to express normal behaviour
- Freedom from fear and distress

The five freedoms essentially take a broad-based approach to ensuring animal welfare is maintained, and to some extent aspects of feelings-based approaches, function-based approaches and comparisons between wild and captive animals' behaviours can be incorporated into these criteria. This approach therefore effectively serves to encompass aspects of all three of the welfare approaches, and as suggested by Fraser (2008), they validate each point of view on defining animal welfare.

### **The vertebrate stress response**

The stress response is initiated after the perception of a threat or a challenge to homeostatic balance by the animal (Sherwood, Klandorf & Yancey, 2005; Keeling & Jensen, 2002). In mammals, the stress response involves a complex suite of physiological actions, integrated by the hypothalamus (Fig. 1.1). Following the perception of a stressor (such as the presence of a predator) by the animal, the sympathetic nervous system (SNS) is activated. This results in increased cardiac output and ventilation as well as redirection of blood flow to skeletal and cardiac muscle, whilst a simultaneous increase in adrenaline excretion from the adrenal medulla rapidly reinforces such physiological changes, which prepare the body for immediate action.

The CRH-ACTH-glucocorticoid system (more commonly referred to as the hypothalamic-pituitary-interrenal (HPA) axis), is also initiated in response to acute stressors. Increased levels of corticotropin releasing hormone (CRH) cause the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary (ACTH). An increased blood level of ACTH causes production and release of corticosteroid hormones from the adrenal cortex. Cortisol and corticosterone have important roles

in glycogenolysis (thus liberating glucose reserves from the liver's glycogen stores), plus mobilisation of amino acids for facilitation of gluconeogenesis. Additionally, modulation of insulin and glucagon secretion (increases in glucagon and decreases in insulin) further reinforce the body's ability to make use of its glycogen reserves to provide energy (Lee, 1992).

The SNS also activates the renin-angiotensin-aldosterone system (RAS), following decreased renal blood flow as a result of vasoconstriction to the kidneys. This hormonal pathway maintains plasma volume and electrolyte levels, whilst increased vasopressin secretion by the posterior pituitary gland reinforces also assists in the maintenance of blood volume (Reeder & Kramer, 2005). (See Fig. 1.1 for summary of the vertebrate stress response.)

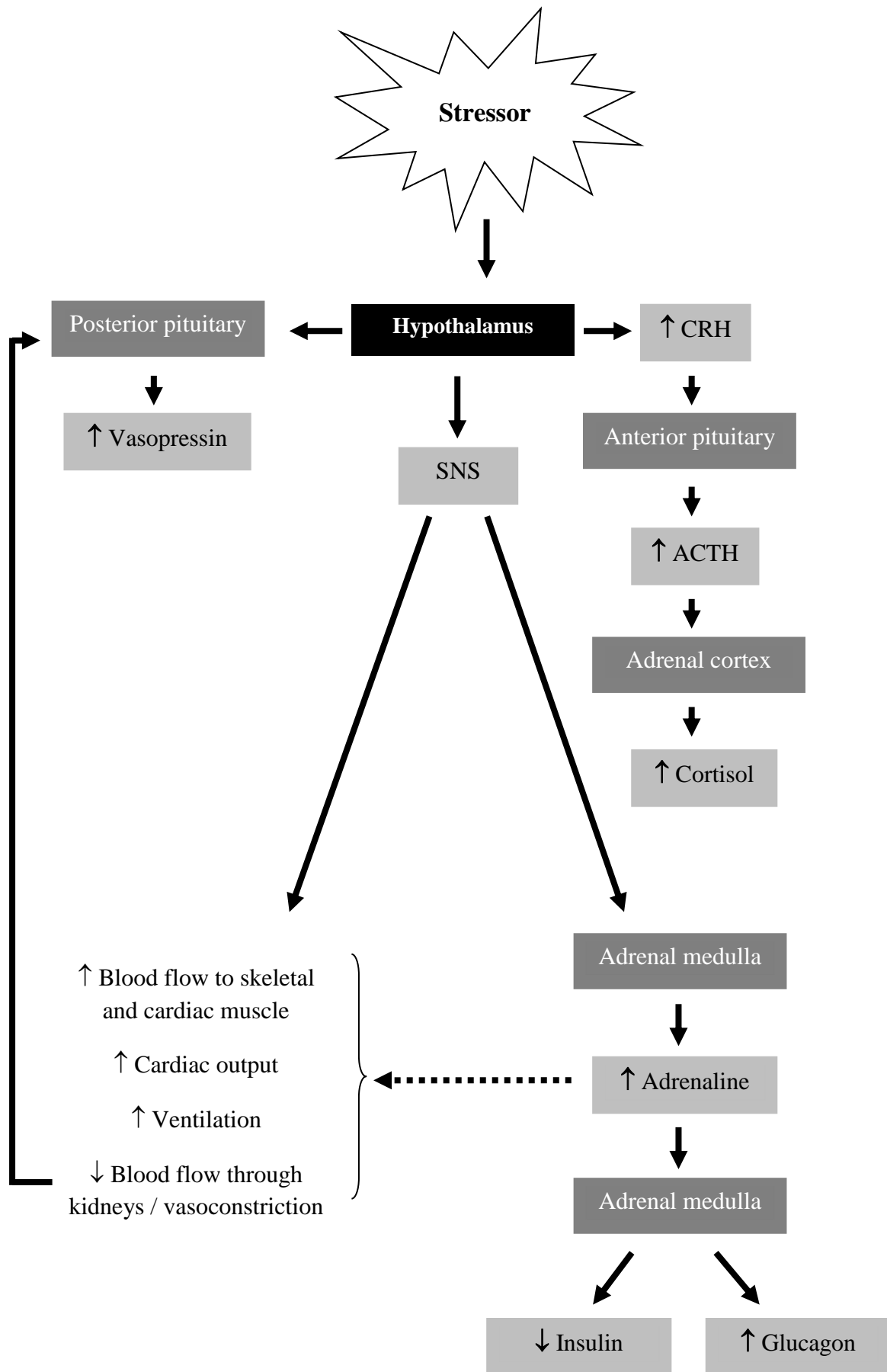


Fig. 1.1: Summary of the vertebrate stress response (SNS = sympathetic nervous system)



## **The teleost stress response**

The neuroendocrine stress response is highly conserved across the vertebrates. Consequently, in bony fish (teleosts), the stress response is comparable with the mammalian system with some anatomical differences. Stress in fish has been considered in terms of a primary, secondary and tertiary response (Fig. 1.2; see also Barton, 2002).

As is the case for mammals, two mechanisms are activated as part of the primary stress response in fish; the sympathetic nervous system which results in the release of catecholamines from the chromaffin tissue around the kidney, as well as corticosteroids being produced from the hypothalamus-pituitary-interrenal axis (HPI), analogous to the mammalian HPA axis. There are fundamental similarities between the teleost and mammalian stress responses, with the hypothalamus produces CRH causing the release of ACTH from the anterior pituitary upon the detection of a stressor. ACTH acts upon the specific kidney tissues (interrenal cells of the head kidney) to produce corticosteroids such as cortisol. Adrenaline or noradrenaline are also secreted and act to increase heart rate and blood pressure alongside an increase in blood glucose concentrations and metabolism as a result of increased cortisol that prepares the animal for action. These potent biochemicals have immediate effects on the body, triggering the next phase of the stress response (Pottinger, 2008).

The secondary stress response in teleosts sees rapid physiological changes taking place which consolidate the effects of the primary response: the respiratory rate is increased, blood flow to the gills is maximised through increased vasodilation, and the heart stroke volume increases. These effects, coupled with fat and carbohydrate stores being mobilised and increased alertness being shown, further prime the fish for immediate action (Wendelaar Bonga, 1997).

If stressors are experienced for a prolonged period of time, or severely stressful experiences are encountered frequently, the tertiary stress response can be initiated. As a result of extended exposure to cortisol, some further physiological effects are

experienced; growth and reproduction are suppressed; the immune system is also suppressed, and disease resistance is reduced. Chronically stressed fish will tend to show a general lack of condition, as well as increased levels of parasitic and other diseases (Conte, 2004). The tertiary response also initiates pronounced changes in the ability of the fish to osmoregulate due to the influence of stress hormones on gill, and possibly skin, permeability (Wendelaar Bonga, 1997; Barton, 2002; Pottinger, 2008).

#### *The adaptive nature of the stress response in fish*

The various stages of the stress response may be seen in captive fish, and it is widely accepted that stress significantly contributes to disease and mortalities in aquaculture (Ashley, 2007; Barton, 2002) and the ornamental fish trade (Crosby *et al.*, n.d.). However, an acute stress response is adaptive, as it prepares the fish for immediate action in appropriate situations, such as during the threat of predation, territorial disputes with conspecifics and heterospecifics, as well as during competition for mates (Pottinger, 2008). Clearly, the evolution of such a response across the diverse taxa represented by teleosts and its retention throughout vertebrate taxa in general in evolutionary terms must have survival value. Rapid mobilisation of fat and carbohydrate reserves is essential for the escape response, as is increased vascular activity due to the requirement for increased oxygen supply to skeletal muscle. On a short-term basis, and seemingly paradoxically, the temporary shutdown of the immune response, plus the effects on the reproductive system and growth, are also adaptive. A temporary suppression of such biological systems diverts energy and essential resources elsewhere where they may be utilised as immediately required. Additionally, it has been suggested that due to the potent nature of many of the hormones involved in the stress response, immunosuppression is necessary to prevent auto-immune reactions by the fish to its own neuroendocrine responses (Råberg *et al.*, 1998).

However, whilst the primary and secondary stress responses may be experienced in wild fish due to short-lived stressors, and such responses are adaptive in nature, captive fish may experience prolonged stressors (which may not be encountered in nature), and these which can initiate the tertiary response. Such stressors include

repeated handling; overcrowding; competition for food; inappropriate tank mates; unsuitable lighting intensity and / or photoperiod and inappropriate furnishings in the aquarium environment. Additionally, due to the intimate relationship between fish and their aquatic milieu, many environmental parameters, if deviating from often narrow ideal boundaries, may cause stress. These include the presence of nitrogen-based waste products (ammonia, nitrite and nitrate); pH; phosphates and temperature. Whilst the stress response of teleosts has evolved to enable them to maintain homeostasis in natural short-term scenarios, initiation of tertiary responses in response to, for example, lack of enrichment; repeated handling; high stocking densities (Bolasina, 2006); acute infection (Ellis *et al.*, 2007); or elevated nitrates in captivity reflect the disparity between the nature of wild and captive stressful situations for these animals (Pottinger, 2008; Barton, 2002).

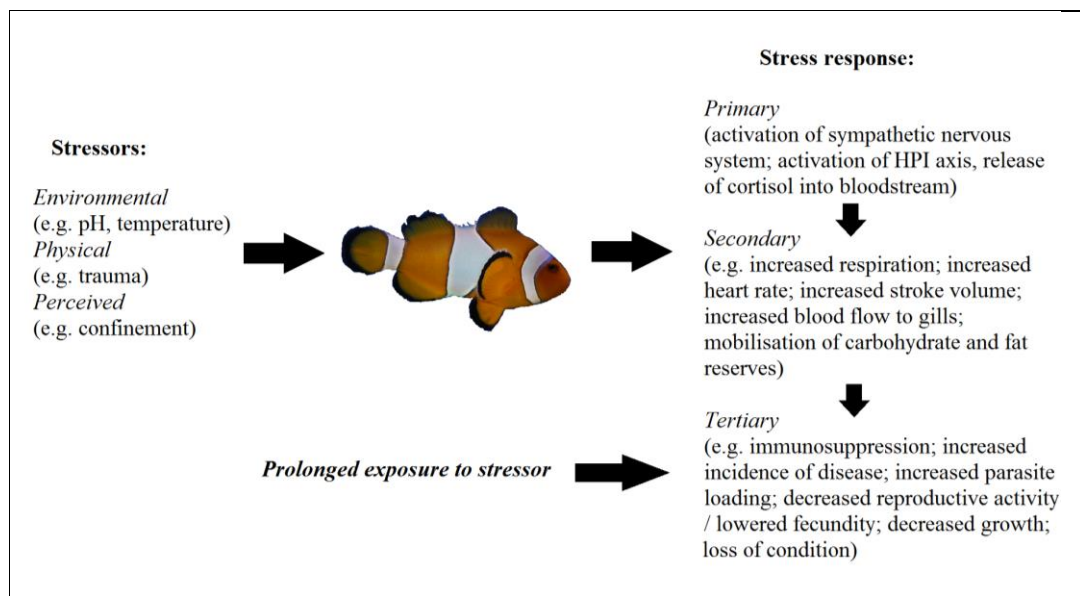


Fig 1.2: Summary of the teleost primary, secondary and tertiary stress responses

### Measurement of stress in fish

There are a range of techniques available for quantifying the stress response in fish. The economic importance of the salmonids, and the negative impact that the stress response may have in terms of growth, fecundity and flesh quality means that a wealth of literature has been published on this taxon, with many other groups the focus of further study (Pottinger, 2008).

Aspects of the primary response which may be used include gene expression of, for example, messenger RNA (mRNA) influencing the HPI cascade (Doyon, Trudeau & Moon, 2005), as well as pituitary hormone assay, for example prolactin (PRL) (Pottinger, Prunet & Pickering, 1992). However, cortisol widely recognised as being a reliable indicator of the initiation of, and even the degree of severity of, the primary stress response in fish (Barton & Iwama, 1991).

Secondary stress responses may be quantified through behavioural means. For example, Olla, Davis and Schreck (1992) studied the ability of juvenile coho salmon (*Oncorhynchus kisutch*) to avoid lingcod (*Ophiodon elongatus*) following a stressor (in this case handling). The results suggest that a short (up to 90 minute) period is experienced by stressed coho salmon in which predator avoidance is compromised. Further measures of the secondary stress response in teleosts include plasma glucose and lactate concentrations (Wells & Pankhurst, 2007) and determination of the quantity of circulating lymphocytes. However, Pickering and Pottinger (1985) suggest that agents such as cortisol may mediate the stress response without an apparent decrease in lymphocyte count, calling into question the validity of examining white blood cell counts in isolation as an indicator of stress.

The tertiary stress response (i.e. the long-term response to prolonged exposure to stressors) may be evaluated through a variety of physiological and behavioural responses, with developmental performance such as growth and reproduction also being a measurable variable (Weerd & Komen, 1998; Schreck, Contreras-Sanchez & Fitzpatrick, 2001). Additional factors such as disease and mortality may be useful measures of the long-term effects of stress on teleosts (Pickering & Pottinger, 1989).

One of the most widely-used and robust indicators of stress in fish is the steroid hormone cortisol (Wendelaar Bonga, 1997), due to its relative ease of measurement and relatively reliable data generated through its assay, with baseline levels having been established for many species against which increases may be compared in experimental settings.

## Measurement of cortisol

Measurement of cortisol can be performed in plasma, whole body or water samples (see Pottinger, 2008 for a summary), with techniques varying in terms of their degree of invasiveness, and therefore the degree to which they may elicit a stress response themselves. Handling, air emersion and anaesthesia to obtain a blood sample are all potential stressors; therefore, studies must be carefully designed and conducted. Humane and rapid killing of fish for whole body measurements are essential but this technique can only be applied to small species that can be killed and frozen rapidly (Pottinger, T.G., personal communication, April 2011). If conducting a time series study and using very small individuals, terminal sampling is required at each time point thus increasing the number of fish required for experimentation. Measuring cortisol in water samples, therefore, may provide a non-invasive means of obtaining the stress responses of fish but could also reduce numbers by making it easy to obtain several samples from the same individual thereby reducing the numbers of animals used.

### *Invasive measurement of cortisol: cortisol in blood and plasma*

Collection of cortisol from the blood or plasma offers the most powerful technique to infer the 'real time' stress status of fish. Blood may be taken from anaesthetised fish using a variety of techniques, including extraction from the caudal vein using a needle and syringe; cutting the tail off humanely-dispatched fish and collecting the blood into tubes or a heparinised capillary tube; via the large blood sinus (*ductus Cuvieri*) at the posterior of the opercular cavity housing the gills; or via heart puncture (Ostrander, 2000). However, this approach clearly presents challenges and disadvantages. For example, the sampling technique itself is inherently stressful, and samples must be taken within just a few minutes to prevent the stress from sampling itself from elevating plasma cortisol levels (Pottinger, T.G., personal communication, July 2011). Additionally, whilst this technique has been successfully utilised to determine the stress levels of large fish such as adult rainbow trout (*Oncorhynchus mykiss*) without the need to sacrifice the individual, smaller fish species such as three-spined sticklebacks (*Gasterosteus aculeatus*), or juveniles of

larger species yield such small volumes of blood for assay that ‘one-off’ sampling only is possible, resulting in the unavoidable death of the individual concerned.

#### *Invasive measurement of cortisol: cortisol in bile*

Cortisol may be sampled from the gall bladder due to hepatic metabolism of the hormone, although this provides a less immediate and apparently less exact method of assay than from blood and plasma. Studies have shown that there is a lag between the onset of the stress response in sampled fish and the appearance of cortisol and steroid conjugates within biliary isolates. For example, in rainbow trout subjected to stressors, elevated free cortisol was detected 2 hours after appearance within plasma samples (Pottinger, Moran & Cranwell 1992). Four hours after the stressful event, conjugated steroids of various types were detected in the gall bladder. Therefore, gall bladder samples, as well as being invasive, require that assay techniques take into account the variety of immunoreactive cortisol conjugates present in addition to free cortisol itself.

#### *Invasive measurement of cortisol: cortisol in whole-body homogenates*

The use of whole-body homogenates can be a relatively reliable technique for determining the levels of cortisol and cortisol conjugates in small fish species when blood and plasma sampling is not practical, or else blood sampling will yield volumes too small for assay. Humanely-euthanised fish are homogenised and the resulting supernatant may then be used to determine steroid levels. However, whole-body homogenates not only include cortisol concentrations from the blood, but they also include cortisol and cortisol conjugates from a variety of other tissues (King & Berlinksy, 2006).

#### *Non-invasive measurement of cortisol*

Ultimately, the non-invasive measurement of cortisol presents an opportunity to gain an insight into the stress status of fish in a manner in which the collection technique minimises artificially elevating the stress response, as well as potentially reducing or

completely eliminating the need to sacrifice animals in order to determine cortisol levels.

*Non-invasive measurement of cortisol: cortisol in faeces*

Cortisol and cortisol conjugates are excreted in the faeces of fish, and assay of steroids from faecal material has wide-ranging potential to offer insights into the stress levels of fish within the confines of aquaria, as well as within open caged systems and even in wild environments, as suggested by Turner, Nemeth and Rogers (2003). Three species of parrotfish, stoplight (*Sparisoma viride*), queen (*Scarus vetula*) and rainbow (*Scarus guacamaia*), were maintained in aquaria to obtain baseline hormone data from faecal material, and these data were compared with faeces collected from wild parrotfish on the reef. The results suggested that this novel technique may be a useful way in which the stress response may be evaluated relatively non-invasively in environments in which such data are challenging to collect, although it should be noted that linking deviations from baseline cortisol levels in samples collected in such a manner to specific events (stressful or otherwise) perhaps presents numerous challenges. It should be noted that, according to Ellis, James and Scott (2005), the vast majority of free steroids released by a fish are released via the gills, with their conjugates being released via the urine and faecal material. Scott and Ellis (2007) suggest that the use of steroid conjugates collected from fish, which this technique would be presumably facilitate, will be subject to time lags dependent on factors such as conjugation rates and feeding activity. Nevertheless, such methods could yield valuable medium- to long-term information on fish stress levels.

*Non-invasive measurement of cortisol: cortisol measurement from water*

Cortisol and a range of other steroids are also passively diffused by fish via the gills, due to their apparently high permeability to such molecules. The extensive vascularisation and permeability of the gill lamellae means that significant quantities of cortisol may be released to the water in this manner down a concentration gradient (i.e. from relatively high concentration within the fish's bloodstream to lower concentration within the surrounding water) (Scott & Ellis, 2007). Additionally, loss

of free cortisol across the gill membrane is pronounced in fish due to their lack of proteins which bind cortisol (such as transcortin, which is responsible for binding significant proportions of cortisol and other hormones in many other vertebrate groups).

Measurement of cortisol in the water presents an opportunity to quantify the stress response in a non-invasive manner since it is usually measured in blood samples that require terminal sampling in small individuals, or the use of whole body homogenates. Measurement of water-borne steroids has been validated for various species, including the three-spined stickleback and rainbow trout, and published following a variety of experiments (see Scott & Ellis, 2007).

The advantages of collection and assay of fish cortisol and other steroids in water, together with the challenges associated with the use of such techniques, are comprehensively discussed by Scott and Ellis in their 2007 review. They can, however, be summarised as follows.

*The advantages of using water-borne cortisol as an indicator of stress*

The use of water-borne hormone samples allows for non-invasive measurement, and thus the potential for the experimental technique to play a minimal part in causing a stress response, which may otherwise bias results. Repeated sampling of the same fish is possible utilising such methods, whereas for collection of whole-body homogenates or plasma samples from small species, sacrifice of the animal is necessary. Additionally, it is suggested that integration of the stress responses from multiple fish may be measured, through sampling systems in which more than one animal is maintained. However, caution may be necessary when inferring from multiple subjects, as there appears to be considerable variation in the susceptibility of individual animals including fish to various stressors, with extensive evidence for ‘personality traits’ such as ‘bold’ and ‘shy’, with associated genetic and behavioural profiles that are related to stress coping style or differential cortisol production during stress (Sneddon, 2003<sub>b</sub>; see also Pottinger and Carrick, 1999). Intraspecific variations in behavioural traits have recently been noted in invertebrate subjects as diverse as hermit crabs *Pagurus bernhardus* (Briffa, Rundle & Fryer, 2008) and



even sea anemones *Actinia equina* (Briffa & Greenaway, 2011). In spite of the variability seen between individuals in such diverse taxa, it seems plausible, however, that integration of the stress response from multiple subjects is valid if repeated sampling of the same individuals is carried out in the same closed system, with baseline data being generated for each group of fish. Consequently, elevations in the total cortisol from the group can infer a collective stress response, although it may not be possible to determine the contribution of each individual to the total cortisol measured.

Scott and Ellis (2007) note that polar organic chemical integrative samplers (POCISs), currently being used primarily for monitoring of wastewater, offer the possibility of acting as a means of collecting hormonal data over time (Alvarez *et al.*, 2007). These devices can be placed in an aquatic environment, adsorbing excreted steroids passively for subsequent assay. The development of such technology for use in this context is currently at a nascent stage, although future refinements may allow for practical applications of such methods in non-invasive measurement of stress in a variety of closed or semi-closed aquatic systems.

It has also been suggested that the development of a ‘dip test’ or other practical method of rapidly measuring the cortisol excretion by fish may be a valuable tool for immediately assessing the stress levels of individual fish or groups of fish (Scott & Ellis, 2007). Presumably, if such a technique could be devised, based upon a test strip perhaps using a sandwich enzyme-linked immunosorbent assay (ELISA) method, the cortisol concentration in a given water sample could be compared to baseline data, and conceivably also compared to previously-established elevated cortisol levels under a variety of stressful conditions. Such data has already been published for many commercially-relevant species in aquaculture, such as rainbow trout (Ellis *et al.*, 2004) and common carp (*Cyprinus carpio*, Lower *et al.* 2005). As with the present status of the POCIS as means of non-invasive hormone assay, however, such technology seems several years away as a means of assaying hormonal release from fish, and sampling of water and subsequent isolation of hormones using Solid Phase Extraction (SPE), followed by ELISA or Radioimmunoassay (RIA) remain the current ‘state of the art’.

*The challenges and disadvantages of using water-borne cortisol as an indicator of stress*

The low rates of cortisol release from some fish, particularly small specimens sampled for a short period of time, mean that very low amounts of cortisol may be accumulated for assay. This poses problems for hormone measurement, with highly sensitive techniques being required for accurate assay. Although ELISA techniques may demonstrate insufficient accuracy to yield meaningful results in practice with very low yields of cortisol (Pottinger, T.G., personal communication, April 2011), RIA is highly sensitive, and capable of detecting within a few pg per sample with accuracy.

Scott and Ellis (2007) report that problems have been encountered with instability of steroids during extraction due to the choice of solvents used for elution of samples from SPE cartridges, and advise caution, stating that the purity of reagents may not necessarily correlate with their increased suitability for purpose. Specifically, they cite issues with ultra pure diethyl ether (which had been stabilised in copper foil) interfering with cortisol extraction. Despite switching to analytical grade diethyl ether in ethanol and experiencing improved cortisol recovery, they recommend the use of ethyl acetate as a suitable elution solvent.

Validation of water-borne cortisol excretion is important to determine the significance of the technique, specifically with regard to correlating the values yielded from measurement in water to the levels found within the plasma. Sufficient numbers of fish are necessary in such studies to mitigate the effects of individual variation in the stress response (Pottinger, T.G., personal communication, April 2011).

The length of time for which steroids are stable in the water sample is also important to consider. The rate at which cortisol is metabolised may be, to a certain extent, temperature dependant. For example, Sorenson et al (2000, cited by Scott and Ellis, 2007) determined the half life of both androstenedione (Ad) and 17 $\alpha$ , 20 $\beta$ -dihydroxypregn-4-en-3-one (17,20 $\beta$ -P) to be 6 hours at 18°C. However, according to Ellis *et al.* (2004), cortisol appears to have a half life of 16 hours at 12°C. It is still

unclear the extent to which these variations in stability are the result of the tertiary structure of the hormones themselves, their metabolism and / or assimilation by microbial communities, the effects of water temperature, or combinations of these factors. Nevertheless, cortisol appears to be relatively stable in water, and it may be presumed that lower temperatures equate to greater stability, evidenced by the long-term storage of cortisol being possible in samples subjected to freezing for many months.

It is suggested that excreted steroids may be subject to re-uptake by fish, and therefore caution should be observed when interpreting the results of studies in which the results are derived from the integration of cortisol excretion of multiple individuals in recirculating systems. However, studies are sparse to date on the full effects of the bioaccumulation of hormones 'acquired' from conspecifics. For example, although Maunder et al (2007) report that three-spined sticklebacks appeared to take up  $17\beta$ -oestradiol and testosterone added to system water, Martins, Eding and Verreth (2010) suggest that non-stressed Nile tilapia (*Oreochromis niloticus*) placed with previously-stressed individuals in recirculating systems appeared to be unaffected in terms of feeding behaviour, which may call into question the significance of re-uptake of the cortisol from stressed conspecifics. Clearly, further research is needed to evaluate the mechanisms by which water-borne hormones may be bioaccumulated by fish, and their subsequent effects.

The interpretation of cortisol excretion based upon fish size is apparently inconsistent within the literature, and there may be significant variances in the apparent meaningfulness of results when factors such as this are taken into account. Whilst it may be true that relative gill surface area decreases with size (as demonstrated in the walleye *Stizostedion vitreum*, Rombough and Moroz, 1997), and thus the brachial surface available for excretion of hormones such as cortisol decreases with age or increasing size, the extent to which this may be factored in to interpretations of cortisol assays is not clear. Nevertheless, many researchers consider fish weight as an important factor when interpreting cortisol release from fish.

## Issues affecting fish welfare

As suggested by Sneddon (2011), in commercial environments such as deep sea trawling, fish may be subjected to practices that would be deemed intolerable for other vertebrates, although the numbers of individuals exploited in, for examples, fisheries alone, far outnumber those of terrestrial animals farmed for meat. The following represent some of the major practices which may compromise welfare of fish.

### *Aquaculture*

There are a range of welfare issues presented by aquaculture, which are summarised in Ashley's 2007 review. These include the following:

*Exposure to unnatural stocking densities:* Many studies indicate that stocking density can have a negative impact on the welfare of fish, and as discussed by Turnbull *et al.* (2008), water quality and the effect of social interaction are closely linked to the welfare of farmed fish. However, the situation is a complex one, and further studies are needed to determine the true impact of stocking density on fish welfare. For example, Ellis *et al.* (2005) suggest that both high and low stocking densities may compromise welfare. It is suggested that high stocking density and the resulting sub-optimal water quality may cause decreased growth and lack of body condition as well as physical symptoms such as fin erosion. Fin erosion, often termed fin rot in an aquacultural context, refers to loss of, or damage to, the tissue of the fins of fish, and is a common problem affecting farmed salmonids – see Ellis *et al.* (2008).

*Exposure to sub-optimal water quality:* The range of water quality parameters which can have deleterious effects on fish in aquaculture have been extensively evaluated, due to the high economic value of such fish. Salmonids in particular appear to be the focus of research (MacIntyre *et al.*, 2008), although numerous other important food fish from other taxa are also the focus of study with regards to the effects of water quality.

The effects of ammonia have been studied extensively, and whilst ammonia is certainly toxic to aquatic life (Tomasso, 1994), it appears that many factors influence the effects of this primary waste product on fish and much of the literature appears to be contradictory in its findings. Factors influencing the chronic or acute toxicity of ammonia include temperature (Thurston & Russo, 1983) and pH (Thurston, Russo & Vinogradov, 1981), both of which influence the proportion of ionised ( $\text{NH}_4^+$ ) or toxic unionised ammonia ( $\text{NH}_3$ ) present. Additionally, it appears that fish may become acclimated to ammonia, with physiological 'defence mechanisms' initiated. For example, Russo and Thurston (1991) revealed that rainbow trout could acclimate to elevated ammonia levels, and Redner and Stickney (1979) similarly suggest that the blue tilapia (*Oreochromis [Tilapia] aureus*) were able to adapt to unusually high ammonia levels with associated histopathological changes noted in response.

Nitrite ( $\text{NO}_2^-$ ) is produced by nitrifying microbial communities within biological filtration in aquacultural systems, and it has known deleterious effects, especially in freshwater fish. The toxic effects of nitrite are primarily related to its ability to bind with haemoglobin (Hb) in the gills, creating methaemoglobin (metHb), rendering the respiratory pigment unable to bind oxygen and leading to suffocation of the fish. The toxicity of nitrite varies in conjunction with the presence of chloride ions, as these competitively inhibit the transport of the nitrite ion across the chloride secretory cells, although a 96 h  $\text{LC}_{50}$  (median lethal dose) of just 0.24 mg/l has been reported for rainbow trout (Lewis & Morris, 1986).

Nitrate ( $\text{NO}_3^-$ ), the end product of the nitrification process in aerobic biological filtration, is widely regarded as being of minor importance in aquacultural systems due to the relatively low levels of this ion in relation to the levels observed to have deleterious effects. According to McGurk *et al.* (2006), 96 h  $\text{LC}_{50}$  data for swim-up fry of lake trout (*Salvelinus namaycush*) reveal that levels of over 1000 mg/l cause acute toxicity, whereas earlier life stages appear to be more sensitive.

*Handling:* In aquaculture, fish may be exposed to handling stress, and this may be repeated a number of times for each individual, for grading, vaccination, slaughter, etc. Handling has been shown to elicit a stress response in numerous studies, for example in the red sea bream *Pagrus major* (Biswas *et al.*, 2006), Atlantic salmon

*Salmo salar* (McCormick *et al.*, 1998) and common carp *Cyprinus carpio* (Saeji *et al.*, 2003). Minimal handling and the development of handling techniques which limit stress will assist in the improvement of fish welfare.

*Exposure to infectious diseases:* Numerous pathogenic diseases may affect farmed fish, including those caused by the following agents:

- *Bacteria:* (e.g. *Renibacterium salmoninarum*, responsible for bacterial kidney disease in salmonids.) (Cefas, 2011)
- *Parasites:* (e.g. sea lice, crustaceans of the Family Caligidae, which may be a significant problem in the salmonid farming industry.) (Costello, 2009)
- *Viruses:* (e.g. infectious salmon anaemia virus (ISAV), responsible for infectious salmon anaemia in salmonids.) (Falk *et al.*, 2013)
- *Fungi:* (e.g. *Saprolegnia* and other oomycetes which may affect a variety of fish species.). (Bruno, Van West and Beakes, 2011)

The range of diseases affecting teleosts is vast, and they may be precipitated by, and their effects exacerbated by, stressors such as poor water quality, excessive handling and crowding. Management of factors such as water quality assist in the management of disease, thus promoting welfare, although chemical control of many pathogens is widely practised in the event of outbreaks. Additionally, vaccination is now commonplace for many diseases and is a vital aspect of modern salmonid farming (Sommerset *et al.*, 2005). Biological control has also been successfully trialled against certain parasitic diseases, notably through the use of cleaning wrasse, primarily the goldsinny (*Ctenolabrus rupestris*) to control sea lice in salmonids (Costello, 1993).

*Transport:* In the UK, legislation is in place which regulates the transport of animals, and this applies to the transport of fish as well as mammals. The legislation includes The Welfare of Animals (Transport) (Scotland) Regulations 2006; The Welfare of Animals (Transport) (England) Order 2006 and The Welfare of Animals (Transport) (Wales) Order 2007.



Fig. 1.3: Fish transport vehicle with GRP holding tanks, each equipped with air diffusers linked to a compressor to ensure adequate oxygenation during transit. *Photo by author*

The stress response of fish to transport appears to vary according to the technique used. Whereas road transport (Fig. 1.3) may elicit measurable stress due to confinement and deterioration in water quality, Erikson, Sigholt & Seland (1997) suggest that the use of modern well boats for the transport of Atlantic salmon – an established practice in Norwegian salmonid fisheries – results in various physiological parameters typically used in measurement of stress (including muscle tissue pH and adenylate energy charge) not changing significantly from those in unstressed fish. This is explained by the well boat's ability to maintain optimal water quality, as well as the rapid netting facilitated at the place of slaughter.

### *Fisheries*

Fisheries exploiting wild populations of fish employ techniques which may significantly compromise fish welfare. These are discussed by Sneddon and Wolfenden (2012) in *Sea the Truth*, and include the following:

*Capture methods:* The range of techniques utilised to capture fish can lead to significant stress and pain. These include the use of hook and line as in long lining (Clearwater & Pankhurst, 1997), trolling and pole-and-line (FAO, n.d.<sub>a</sub>), as well as netting techniques such as trawls (Suuronen 2005), gill nets (FAO, n.d.<sub>b</sub>) and seines.

*Bycatch discards:* The discarding of non-target species ('bycatch') is a controversial practice, necessitated in many countries due to quota enforcement. Bycatch fish may suffocate on board the vessel and / or be subject to the injuries described above as a result of capture (Metcalf, 2009). The use of bycatch discards is wasteful, and in many cases, perfectly marketable fish are thrown overboard; on welfare grounds alone, banning the discard of bycatch would influence an improvement in welfare – however, there are also proven positive impacts for the overall health of fish populations as a result of prohibiting discards. Trials of discard bans (for example, the Norwegian ban of discards in Northeast Arctic waters) resulted in recovery in fish stocks, and despite the initial dip in economic return, the market soon adapts to the more diverse range of species captured (Diamond & Beukers-Stewart, 2011).

*Post-capture practices:* There are considerable variations in the techniques employed in fisheries to dispatch animals. These range from simply allowing the fish to suffocate on the boat's deck which may take up to 250 minutes (fishcount.org.uk, 2013), to live gutting. Each of the techniques presents a challenge to the welfare of the fish concerned, although research is currently lacking in the welfare status of subjects from wild fisheries during slaughter compared to their farmed counterparts.

### *Experimentation*

Fish maintained in laboratory facilities, for example the zebrafish, *Danio rerio*, which has become a widely-used model for genetic and physiological research in vertebrates (Lawrence, 2007) and the pufferfish, *Takifugu rubripes*, which is the subject of considerable genomic sequencing research, may be subject to husbandry practices which compromise welfare. For example, the very nature of laboratory aquarium systems dictates that furnishings are kept to a minimum to maintain 'clinical' conditions (Sneddon, 2011); fish may be maintained in confined conditions



in aquaria (FSBI, 2002); high stocking densities may lead to elevated stress responses (Ramsay *et al.*, 2006); and handling has been shown to invoke heightened levels of cortisol in such fish (Ramsay *et al.*, 2009).

Nevertheless, fish are becoming increasingly popular as the subjects of laboratory studies, in particular zebrafish, with the embryonic stage of the fish gaining acceptance as an alternative to other vertebrate models in various branches of the biosciences, from genetic research to ecotoxicology (Strähle *et al.*, 2012). Clearly, the benefits of using fish in research are considerable to humans, from cardiac disease study to the development of new drugs (Lieschke & Currie, 2007). Whilst EU legislation 2010/63/EU places the early, pre-first feed embryonic stages outside the scope of animal welfare legislation, fish post-first feeding are protected under extensive legislation, for example, The Animals (Scientific Procedures) Act 1986 (ASPA) in the United Kingdom (Home Office, 2013). Additionally, research is ongoing which aims to determine the factors leading to reduced welfare in experimental fish, which may inform husbandry practices, improving the welfare of laboratory fish (e.g. Wilkes *et al.*, 2012; Ramsay *et al.*, 2006).

### *The ornamental fish trade*

Fish have become extremely popular as domestic animals, with one in ten UK households now owning at least one ornamental fish (Copping, 2012). In terms of absolute numbers of individuals, fish undoubtedly far outrank every other pet combined. Estimates of the total numbers of pet fish in UK households vary, although OATA, the UK's Ornamental Aquatic Trade Association, suggests that up to 150 million fish are kept as pets in Great Britain (Keith Davenport, personal communication 7<sup>th</sup> June 2013). The total number of fish receiving Common Veterinary Entry Documents at Heathrow Airport's Animal Reception Centre (i.e. those imported to the UK) each year is approximately 35 million (City of London, n.d.) – clearly, the ornamental aquatics trade is a lucrative business. Numerous welfare issues are presented by the ornamental trade in fish, which can be summarised as follows:

*Intensive production:* Ornamental fish production varies greatly in terms of scale. Small facilities may be utilised for production of relatively low numbers of fish in hobbyists' homes, or through the Czech model of small scale 'cottage industry' breeding operations which consolidate fish for export directly to retailers or distributors. Farmed ornamental fish are often produced in large-scale facilities in, for example Israel and the Far East, and these present perhaps the greatest welfare concerns in terms of disease and mortality. For example, often potentially devastating viral outbreaks have been reported from several of these intensive facilities (Chua, 1996; Paperna, Vilenkin & Alves de Matos, 2001).

*Wild capture:* Despite many species being captive bred for the ornamental trade, large numbers of fish are still routinely wild caught. This includes freshwater species, with between 5 and 10% of all freshwater fish being wild caught (Chao & Prang, 2002) as well as marine ornamentals, the vast majority of which are still wild caught due to the challenges associated with breeding these fish. Estimates of the numbers of marine fish entering the trade from captive breeding range from 1% to 10% (UNEP/WCMC, 2003), with a survey of the stock offered from the UK's major distributor of tropical marine fish suggesting that the true figure is closer to the lower estimate. Wild capture from methods such as cyanide fishing (Wood, 2001) clearly compromise welfare, and these have been outlawed internationally although some small-scale cyanide fishing may still persist. Even so, traditional capture methods using nets and stabbing poles may lead to stress, injury and mortalities. Ultimately, wild capture of ornamental fish is a controversial subject due to the issues associated with animal welfare as well as the environmental impact associated with the practices employed and removal of species from their habitat. However, proponents of wild capture cite examples such as Project Piaba, focussing on Brazil's Rio Negro cardinal tetra (*Paracheirodon axelrodi*) fishery, as success stories of how research and management can effect improvements in animal welfare, whilst lessening environmental impacts and providing employment for indigenous people (Project Piaba, n.d.).

*Transportation:* Transport of ornamental fish presents a number of health and welfare concerns, although the extent of these varies depending on various factors, including the species being transported, the length of the supply chain, the total

transport time and the provision of suitable packaging techniques Figs 1.4-1.6). Mortalities in individual shipments vary greatly - Huntingford *et al.* (2006), suggest that very high mortalities (up to 30%) may be experienced from various South American facilities, although these data are perhaps questionable in the context of relatively recent developments in transport practices and initiatives such as Project Piaba and the Marine Aquarium Council (MAC) (Marine Aquarium Council, 2009).

Specifically, fish undergoing transport are generally packed in polythene bags charged with air, and placed in expanded polystyrene boxes which assist in maintaining the temperature of the shipment. Various additives may be used to alleviate the effects of inevitable deterioration in water quality during transport. These include the use of zeolite (aluminosilicate mineral) granules to chemically bind ammonia, or salt to reduce osmotic stress. Nevertheless, fish undergoing transport in this manner may be subjected to increases in ammonia, as well as a build up of carbon dioxide concentrations within the bag. As a consequence of CO<sub>2</sub> excretion, pH may be lowered depending on the buffering capacity of the water, presenting further physiological challenges to the fish. Oxygen levels may fall, although the use of pure oxygen to charge the bag effectively provides sufficient levels to sustain life for the duration of transport (Chow, Chen & Teo, 1994; personal observation).



Fig. 1.4: Typical packing used for air transport of tropical fish. *Photo by author*

Fig. 1.5: Spotted puffer (*Tetraodon nigroviridis*) in transport bag following shipment from the Far East to the UK. *Photo by author*



Fig. 1.6: Captive-farmed silver arowana (*Osteoglossum bicirrhosum*) packed individually for air transport from the Far East in heat-sealed polythene bags. *Photo by author*

## **Conclusions**

Despite a historical lack of research into the topic of fish welfare (and difficulties in generating meaningful data), progress is being made in this area (thanks to highly sensitive techniques such as radioimmunoassay as well as ingenious behavioural studies), and it is now clear that various human activities may compromise the welfare of these animals. Far from being automatons, fish feel pain and may clearly exhibit a measurable stress response following exposure to a variety of stressors. The range of ways in which fish are exploited means they may be exposed to unnatural stressors which elicit negative effects on their health and well-being. The motivation for improvements in fish welfare comes from both ethical considerations as well as from an economic standpoint. Techniques are now available to gather data which may be employed to determine the effects of these stressors, and to potentially inform us of changes which may be made to improve welfare.

The present study attempts to investigate two activities which potentially compromise animal welfare (specifically, the transport of ornamental marine fish and the use of fish in beauty treatments), and seeks to answer the following questions:

1. To what extent is welfare compromised, and which factors may be responsible?
2. To what extent could the results of such investigations be used to potentially modify current protocols or practices, with a view to improving the welfare of the animals?

## **Chapter Two: The Welfare of Ornamental Marine Fish during Transport**

### **Introduction**

The trade in ornamental marine fishes is a highly lucrative global business (worth up to USD330 million per year (UNEP/WCMC, 2003), with over 50 countries contributing to the trade (Marine Conservation Society, n.d.), supplying nearly 1500 species and up to 24 million individual fish annually (UNEP/WCMC, 2003). The transport of live marine fish presents a number of potential welfare issues due to the methods employed potentially causing stress. The industry standard methods for transporting such fish are outlined in the IATA Live Animals regulations (see Walster, 2008). The process involves placing the animal in a plastic (typically polythene) bag, which is partially filled with water (as little water as possible is used to reduce the weight of the shipment and thus lessen freight costs). The bag is charged with pure oxygen to sustain the fish during transport, and then sealed. Individual bags are placed into either expanded polystyrene ('Styrofoam'<sup>TM</sup>) boxes or alternatively in padded boxes utilising air-filled closed cells which provide insulation, thus maintaining temperature during shipping.

Typically, fish are fasted for a short period prior to shipment to reduce the egestion of solid wastes and limit ammonia excretion, in an attempt to lessen water quality deterioration (Lim, Dhert, & Sorgeloos, 2003). Nevertheless, the excretion of ammonia across the gills of the fish leads to an inevitable decline in the quality of the transport water, and may compromise the animal's welfare, as well as leading to potentially serious (and sometimes fatal) health issues. High mortality or 'wipe-outs' can occur with individual shipments due to fish spending a prolonged period of time in transport bags (Walster, 2008; personal observation). As well as the effects of increased levels of nitrogen-based metabolites (primarily ammonia) within the transport water, initial capture and subsequent confinement have been demonstrated to evoke a stress response in fish (Grutter & Pankhurst, 2000; Dhanasiri, Fernandes & Kiron, 2013). The resulting elevated cortisol levels may compromise the health

status of the fish through immunosuppression, as well as having effects on growth, reproductive fitness and overall condition (Pottinger, 2008).

Although there is a considerable quantity of research published which has investigated the responses of aquaculturally-important species to transport (see Schreck, Sanchez & Fitzpatrick, 2001), very few empirical studies have been carried out to determine the physiological and endocrinological effects of transport of ornamental marine fish. Chow, Chen and Teo (1994) examined the physiological effects of simulated transport techniques on the ‘false percula’ or common clownfish (*Amphiprion ocellaris*), noting that pH and temperature were important factors influencing the survival of the fish, with unionized ammonia (NH<sub>3</sub>) also having deleterious effects and Pottinger and Calder (1995) reported that zebrafish received in the laboratory from a commercial supplier exhibited elevated whole-body cortisol levels for up to 24h after arrival. However, overall there is a lack of scientific literature on the stress response of ornamental marine fish undergoing transport.

Quantifying the stress response of teleost fish can be accomplished in various ways. These include the measurement of gene or protein expression in tissues (Iwama, Vijayan, Forsyth & Ackerman, 1999; Krasnov, Koskinen, Afanasyev & Mölsä, 2005), measurement of blood glucose levels (Barton, 2002); and assay of the ‘stress hormone’ cortisol, which in itself can be achieved in a number of ways (Pottinger, 2008). Many of the techniques used for quantifying the stress response are invasive, and they may either invoke a stress response themselves, or else require sacrificing the subject. However, non-invasive measures of stress may be employed, such as behavioural observations (Kane et al, 2004), as well as the sampling of cortisol excreted in the faeces (Karsten, Nemeth & Rogers, 2003) or directly into the water via the gills (Scott & Ellis, 2007). Such techniques offer the possibility to gain insights into the stress status of fish without compromising welfare. In studies so far water cortisol measurements have primarily been validated for freshwater fish thus it would be useful to investigate a marine teleost given their popularity in the ornamental fish trade.

The aim of this study was to investigate the effects of transport on a commonly-imported tropical marine fish. *A. ocellaris* was chosen due to its prevalence in the

trade, high popularity with aquarists (UNEP/WCMC, 2003) and therefore ready availability. Fish were subjected to simulated transport, and their shipping water sampled for cortisol, with validation of the technique by measuring cortisol in whole-body extracts. Additionally, behavioural observations were used to determine whether any links exist between hormonal data and behavioural recovery following transport. Various water quality parameters important for marine fish health (pH, nitrite, ammonia and alkalinity) were measured to determine if water quality deteriorated with transport time and to investigate whether these factors were related to cortisol concentrations. I hypothesised that longer durations of transport will be more stressful accompanied by deteriorating water quality. A second experiment was conducted to explore if improving water quality by the addition of biological filter materials to the transport bag reduced the stress of transport.

## **Methods**

### *Subjects and husbandry*

Common clownfish (*Amphiprion ocellaris*) were held in a centrally-filtered aquarium system (AkvaStabil A/S, Haderslev, Denmark). Wild-caught individuals ( $n = 40$ ; mean weight =  $5.6\text{g} \pm 0.34\text{g se}$ ) were shipped from Indonesia (Tropical Marine Centre, Chorleywood, Hertfordshire, UK). Life support consisted of a sump tank incorporating foam fractionation (protein skimming – TMC V<sub>2</sub> Skim 1500), mechanical filtration (achieved with the aid of 200 $\mu\text{m}$  bag filters followed by filter foam), biological filtration utilising ‘bioball’ plastic media, (Tetra GmbH, Melle, Germany) and chemical filtration through the use of GAC (Granular Activated Carbon, Tetra), which was replaced weekly. Overflows in each of the system’s holding tanks fed water to the sump, and after passing through the filtration system, water was then returned to the holding tanks via an Eheim 1080 pump (Eheim GmbH, Deizisau, Germany). To limit the transmission of pathogens between tanks, a UV steriliser (Aqua Medic GmbH, Bissendorf, Germany) was installed on the return pipework (i.e. following ‘polishing’ of the water post-filtration). The UV system’s quartz sleeves were cleaned monthly, and the UV tube replaced every 6 months to ensure efficient operation as per the manufacturer’s guidance.



The system was maintained at 24°C ( $\pm 1^\circ\text{C}$ ) by heating or chilling as appropriate. Lighting was provided by single T5 high output fluorescent tubes operating on a 10 hour 'day' / 14 hour 'night' timed programme. Salinity was maintained at 34‰ ( $\pm 2\text{‰}$ ) using an 'osmolator' freshwater top-up device administering reverse osmosis (RO) water as appropriate (in order to eliminate increases in salinity due to evaporation of fresh water). Water quality was checked weekly, with ammonia, nitrite, nitrate, pH and alkalinity being measured. Ammonia and nitrite were maintained at  $<0.1\text{mg/l}$ ; Nitrate was maintained at  $<30\text{mg/l}$  nitrate-nitrogen. Alkalinity was maintained at  $3\text{meq/l}$  ( $\pm 0.5\text{meq/l}$ ).

The nine holding tanks (62 x 40 x 42 cm) within the centrally-filtered system were furnished with a shallow ( $\approx 1\text{cm}$ ) layer of fine coral sand and two small pieces of live rock (calcareous rock harvested from coral reefs, comprised of naturally storm-broken coral skeletons) to provide shelter and enrichment for the fish. Water changes of 25% per week were performed on the system, with RO water used as make-up water for salt mixes (Tetra Marine Salt). The fish were fed at 2% body weight per day on Tetra Marine Flakes.

Upon arrival, the fish were allowed to acclimatise to the holding system for two weeks prior to the start of the experiments. Groups of up to six individuals were maintained within the holding tanks. Care was taken to mix fish in order to minimise aggressive interactions (Iwata et al, 2008). Clownfish are protandrous hermaphrodites, with newly-hatched individuals being gender-neutral. As they develop, the fish become subordinate males before perhaps developing to become either a breeding male or (if assuming the highest rank within their group) a dominant breeding female. This complex hierarchical system presents challenges for successful maintenance of groups of clownfish, as aggression between dominant conspecifics can be pronounced (personal observation), and may presumably influence stress levels. Therefore, larger individuals were maintained alongside smaller fish to reduce aggressive interactions in order to minimize social stress from conspecifics.

## *Experiment 1: The impact of transport duration on water quality, cortisol excretion and behaviour*

### *Simulated transport*

For each fish undergoing simulated transport, a single polythene plastic bag was filled with 350ml of the system water. Although the volume of water used to transport ornamental marine fish varies considerably in practice, 350ml was chosen as an approximate average volume for small- to medium-sized marine fish (Rachael Jones, Zoological Society of London, personal communication, November 2010). The bag and water were weighed ( $\pm 0.1\text{g}$ ), and then the fish added, with the total weight of bag, water and fish being used to derive the weight of each individual used in the study. The net used for capture of the fish was carefully selected to aid swift handling and minimise damage to the fishes' protective mucus coating. Fish were bagged individually rather than in groups to determine the cortisol excretion by single fish, rather than integrating the cortisol excretion from two or more individuals. Fish were selected at random for bagging in triplicate groups. According to Lowe-McConnell (1997), *Amphiprion* are semi-gregarious, and therefore three fish were observed post transport rather than individuals. The criteria used for identification were both size and coloration. Following the addition of the fish to its bag, the air was squeezed out and pure oxygen used to inflate the bag before it was tightly secured with a rubber band. Each bag was then placed in an expanded polystyrene box, the lid fitted and the bag left for the appropriate time. All participants wore latex gloves at all times during the procedures to prevent contamination by cortisol excreted through the skin (Ito *et al.*, 2005).

### *Collection of water samples for cortisol assay*

Following the appropriate period of simulated transport, 250ml of the transport water was decanted from the polythene bag, and immediately frozen in a sterile, unused plastic bottle at  $-20^{\circ}\text{C}$  (Green & Leake, 1987) for subsequent assay of cortisol. Each fish therefore remained in 100ml of water following simulated transport. The fish was slowly (over 30 minutes) acclimated to the holding system's water conditions using a slow drip method, introducing a constant trickle of water into the remaining

transport water, thus preventing a rapid shock from rapid alterations in pH, temperature and other key parameters (Harmon 2009). Following the required period of acclimation, and testing to determine that conditions within the transport bag and the holding system were identical using appropriate test kits to measure water quality parameters (ammonia, nitrite and pH), plus a refractometer to measure salinity, the trio of fish were introduced into the holding system for behavioural observation.

### *Behavioural observations*

Behavioural observations were carried out to determine whether the behaviour of fish subjected to transport could be used as indicators of stress. Observations were completed for fish subjected to capture, bagging and immediate acclimation as a control (duration of transport 0 h), as well as those undergoing simulated transport (duration of transport, 0.5, 2, 24, 48 and 72 hours, n = 6 per group).

Four tanks (62cm x 40cm x 42cm) were used for all behavioural observations. The tanks were identically furnished to eliminate the possibility of environmental variation influencing the behaviour of subjects, with a fine coral sand substrate and two small pieces of live rock each. These rocks were arranged to form a small 'cave' offering shelter, whilst still allowing the fish to be observable. The filtration for the observation tanks was achieved with a mature biofilter, maintaining ammonia and nitrite at <0.1mg/l, and nitrate at <30mg/l.

Prior to the experiments commencing, preliminary observations of the fish were undertaken to determine the potential behavioural repertoire of the fish (see Appendix 1). The results suggested that groups of three fish maintained in a holding tank exhibited a more natural behavioural repertoire than those kept individually. An ethogram was devised, and this was employed for all behavioural observations for preliminary analysis (see Appendix 1). The behaviours selected for the main study that were significantly different in an initial analysis were latency to feed (s) and social behaviours (frequency per minute of fish swimming alongside or parallel to a conspecific). A Cohen's Kappa test was carried out to determine that inter-observer reliability between the two observers (D. Wolfenden and K. Magee) was sufficient showing the measurements were robust and valid ( $\kappa=0.85$ ).

Observations were recorded for 10 minutes every hour post transport. Instantaneous sampling was used to record the behaviours of individuals at timed intervals of 30s once the fish were introduced to the observation tanks. Food was offered at the start of each hour in order to determine the latency to feed following simulated transport. In order to reduce disturbance to the subjects, observers wore dark clothing and were positioned 2 metres from the tanks. The observer was present for 15 minutes prior to commencing any observations to allow habituation and the observer did not move from their position until the end of the experiment.

#### *Water sample analysis*

Once collected, the water samples were immediately frozen at below -20°C for subsequent analysis. The samples were defrosted and filtered using 0.45µm nitrocellulose filters (Whatman, Maidstone, UK) placed with a vacuum flask to remove particulate matter following simulated transport. The filtered water then underwent solid phase extraction (SPE) using Sep-Pak<sup>®</sup> C-18 cartridges (Waters, Elstree, UK) via a four-way manifold connected to a peristaltic pump. Priming of the cartridges was achieved through the use of 5ml analytical grade methanol followed by 5ml of distilled water with care taken to ensure that the cartridge did not dry out after conditioning. After each water sample had been passed through the SPE cartridges (at ≈25ml/min), a further 5ml of distilled water was used to purge residual interstitial salts from within the media, and air was passed through to eliminate as much water as possible. (This is necessary to prevent possible splitting of the cartridge during freezing due to expansion of residual water.) Eluate was reserved for subsequent water quality analysis, whereas filtrate was retained within the C18 media (see Fig. 2.1). The C18 cartridges were labelled and frozen, with freezing demonstrated to have an insignificant effect on steroid concentrations, permitting long-term storage of the cartridges (Ellis et al 2004).

Sep-Pak<sup>®</sup> cartridges were eluted and the eluates were assayed for their cortisol content using radioimmunoassay (RIA; Pottinger and Carrick, 1999) at The Centre for Ecology & Hydrology (CEH Lancaster, Lancaster Environment Centre, Bailrigg, Lancaster UK). Blind sampling of cartridges was carried out and assays were validated by assessing the parallelism between a serial dilution of sample extracts

and the assay standard curve, and by determining quantitative recovery. For detailed protocols of SPE and RIA techniques, please refer to Appendices 3 and 4.

Water quality was tested blind at the University of Liverpool, UK, with the following parameters being measured:

- Alkalinity (as milliequivalents/l); total alkalinity (as milliequivalents/l) (Golterman, 1971). Shifts in alkalinity result in pH changes that may cause alkalosis or acidosis resulting in stress, therefore measurement of alkalinity may give insights into potential changes in pH within the transport water.
- pH. Fish must maintain a constant internal pH and acid/base balance in the blood. Disturbed pH can lead to acidosis or alkalosis which is potentially lethal. Most marine fish prefer pH in the range 8.0-8.4, and deviations outside this range may compromise health.
- Total ammonia-nitrogen [TAN] (ppm) (Bran & Luebbe Method no. G-171-96 rev. 10 – Multitest MT19). Ammonia in high concentrations (>0.5mg/L) in the free form is toxic to fish destroying mucous membranes of the skin and intestine, causing external bleeding and internal organ haemorrhaging as well as brain damage. Sublethal concentrations (0.2 – 0.5mg/L) are linked to the prevalence of diseases such as finrot, dropsy and gill disease.
- Nitrite [as nitrite-nitrogen] (ppb); nitrate [as nitrate-nitrogen] (ppm) (Bran & Luebbe Method no. G-172-96 rev. 10 – Multitest MT19). Nitrite causes a phenomenon termed methaemoglobin where the iron in red blood cells is oxidised resulting in a reduction in oxygen carrying capacity and thereby poisoning the fish (toxicity above 5mg/L). Nitrate is much less toxic with many fish species tolerant of 50-300mg/L, however, marine organisms are less able to cope with high nitrate values since in natural marine systems the amount of nitrate is negligible (see review of water quality in Andrews et al. 2002)

## *Validation of water cortisol as a measure of stress*

### *Recovery of cortisol*

Recovery of cortisol from the Sep-Pak<sup>®</sup> cartridges was determined according to the following protocol:

20 ml of stock cortisol in distilled water (1.0 ng/ml) was made by drying down 400 µl of standard solution (50 ng/ml). Samples for extraction were prepared as 250 ml volumes containing 0, 150, 300, 600 or 1200 µl of the cortisol stock solution. Cartridges were washed with 5.0 ml ethyl acetate, primed with 5.0 ml methanol, and wetted with 5.0 ml HPLC grade distilled water. 250ml of distilled water, salt and cortisol were extracted, the cartridges were washed with 5ml distilled water, and 3ml of ethyl acetate was used to wash each cartridge. 250ml of distilled water, salt and cortisol were subjected to extraction. 3ml ethyl acetate were used to elute each cartridge. 2ml of eluate was recovered and dried down, and this was reconstituted in 350µl of ethyl acetate.

500µl of stock cortisol in water (20ng/20ml) was extracted with 500µl of ethyl acetate, and 150µl of this extract assayed (IgG-F2 50µl stock in 12ml buffer, 200µl per tube). [3H] ('hot') cortisol was added in 25µl of buffer after drying down and re-dissolving standards and unknowns. Recovery of known standards were found to be on average 53% so approximately half of the cortisol was recovered from sea water (Appendix 2). However, it was decided that the actual values would be used for analysis in the present study rather than altering the raw data due to the consistency of recovery (41-62% in standards at <600 pg; Appendix 2). Comparison of the assay curve between known dilution of the standard and dilution of unknown samples allowed parallelism of samples to be determined. The resulting assay curves were found to be satisfactorily parallel, indicating no significant matrix effects on cortisol measurement.

### *Whole body cortisol*

After the anaesthetic overdose all fish assayed for water cortisol were frozen at -20°C. Homogenization and extraction of all fish were performed according to the protocol validated by Pottinger and Calder (Pottinger and Calder 1995). Frozen fish

were weighed, then homogenised in 5ml analytical grade ethyl acetate (Sigma) with an IKA T10 Ultra Turrax homogenizer. The homogenate was centrifuged at 1200G (4°C) for 5min and 4ml of supernatant frozen. For analysis, a 100µl aliquot was transferred to a polypropylene tube for the RIA assay as described above.

*Experiment 2: Does improving water quality reduce stress in clownfish during transport?*

In a second experiment, bioballs were added to the transport bags of another set of clownfish to provide denitrifying bacteria that could potentially ameliorate any increases in ammonia and nitrite thereby improving water quality. From the results of Experiment 1 it was clear only transport durations of 24h or more resulted in higher cortisol concentrations therefore to reduce numbers of fish used it was decided to repeat the experiment as described above with transport durations of 24, 48 and 72h (n = 6 each group). Behavioural observations were made subsequently and water collected as previously described.

**Statistical Analysis**

Kolmogorov-Smirnov (K-S) tests were used to determine whether the cortisol, water quality and frequency of social behaviour data conformed to a normal distribution. Due to much of the data assuming a non-normal distribution, non-parametric tests were used to analyse the behavioural data. In order to determine differences over time for each group, Kruskal-Wallis (K-W) tests were performed followed by planned comparison post hoc Mann Whitney U tests with Bonferroni correction for multiple testing. The number of fish consuming food at each time point for each group was analysed using Chi Squared Goodness of Fit test to determine whether there was no effect of transport on the time taken to resume feeding after transport.

The nitrite levels were negligible (all <0.01ppm) thus were excluded from the analysis since this is below the effective concentration to have an effect on fish stress. pH, TAN and alkalinity as well as cortisol concentrations were analysed using Kruskal-Wallis tests followed by planned comparisons (Mann-Whitney with Bonferroni) to determine if these differed with transport duration. As part of the validation cortisol values were correlated with body weight of the fish and it was found there was no effect of body weight thus this does not need to be factored into the cortisol determination (Appendix 3). To explore if there were relationships

between the cortisol values and water quality, pH, TAN and alkalinity were correlated against cortisol using Spearman Rank Correlation.

For experiment 2, similar analyses were conducted as described above to determine whether there was a difference in behaviour, water quality and cortisol with transport duration. Behaviour and cortisol in the bioballs groups and the respective groups from experiment 1 were also compared with one another at each time point to determine the impact of improving water quality.

A significance level of 0.05 and two-tailed tests were used throughout the study and all statistics were carried out on SPSS Version 19.

## Results

*Experiment 1: The impact of transport duration on water quality, cortisol excretion and behaviour*

There was a significant positive relationship between water cortisol and the cortisol in the respective whole-body homogenates ( $R_s=0.987$ ,  $P<0.001$ ,  $n=20$ ; Fig. 2.1) demonstrating the validity of measuring water cortisol as a measure of stress. There was no relationship between water cortisol and body weight ( $R_s = -0.200$ ,  $p = 0.217$ ,  $n = 40$ ; Appendix 3) thus body weight did not need to be factored into the cortisol calculations.

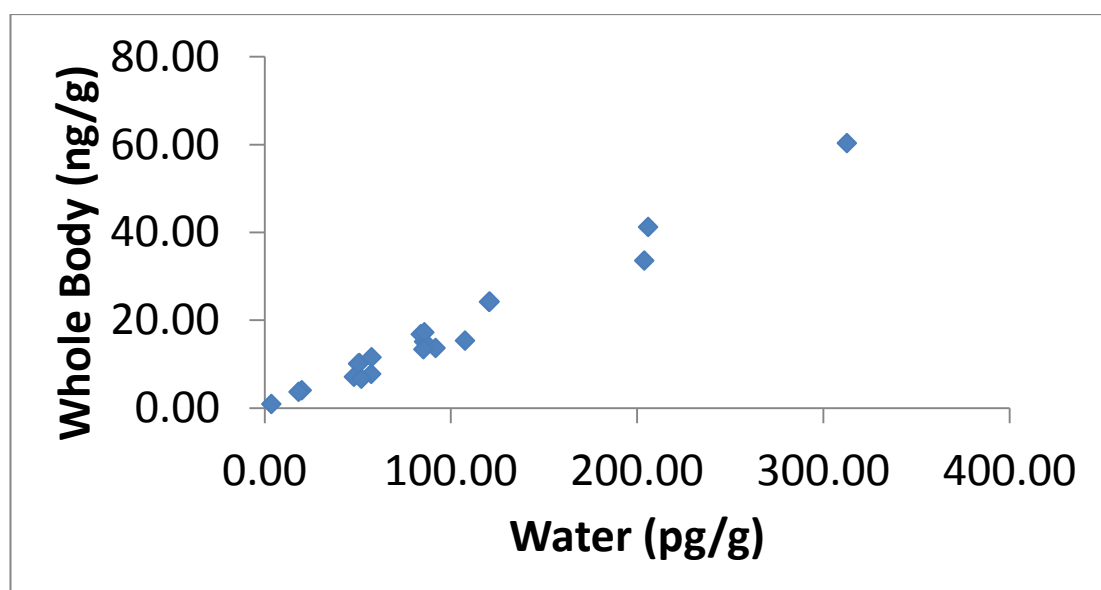




Figure 2.1. The relationship between whole body cortisol (ng/g) and water cortisol (pg/g) in common clownfish ( $R_s=0.987$ ;  $P<0.001$ ;  $n=20$ ).

Whole body cortisol values were highest for 24h and 48 h transport durations ( $H = 10.7$ ,  $P=0.024$ ,  $df = 4$ ; Fig 2.2) compared with acclimation (Fisher's Exact Test 24 h  $P = 0.029$ ; 48 h  $P = 0.030$ ).

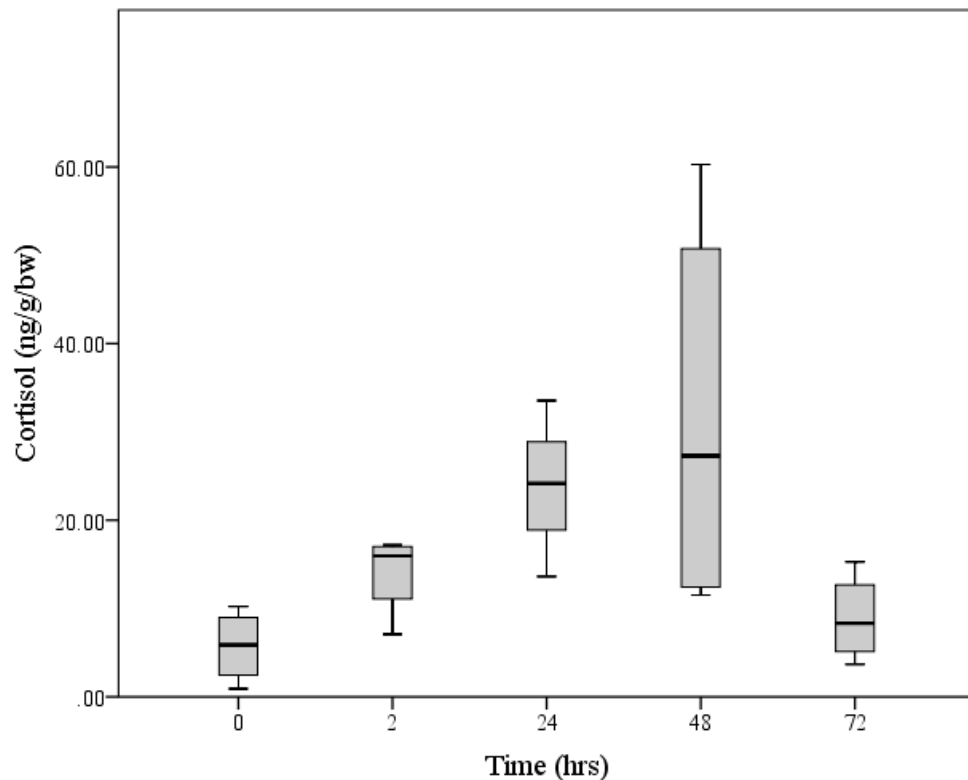


Figure 2.2: Box plots showing median whole body cortisol in common clownfish with increasing simulated transport time from 0 (30 minute acclimation) to 72 hours ( $n = 20$ ).

The mean concentration of water cortisol varied significantly over time, however, there appeared to be a lag in cortisol release with values highest at 48 h and 72 h ( $H = 14.7$ ,  $P = 0.005$ ,  $df = 4$ ; Fig. 2.3). At 48 h ( $P = 0.015$ ) and 72 h ( $P = 0.045$ ) values were significantly higher than 24 h.

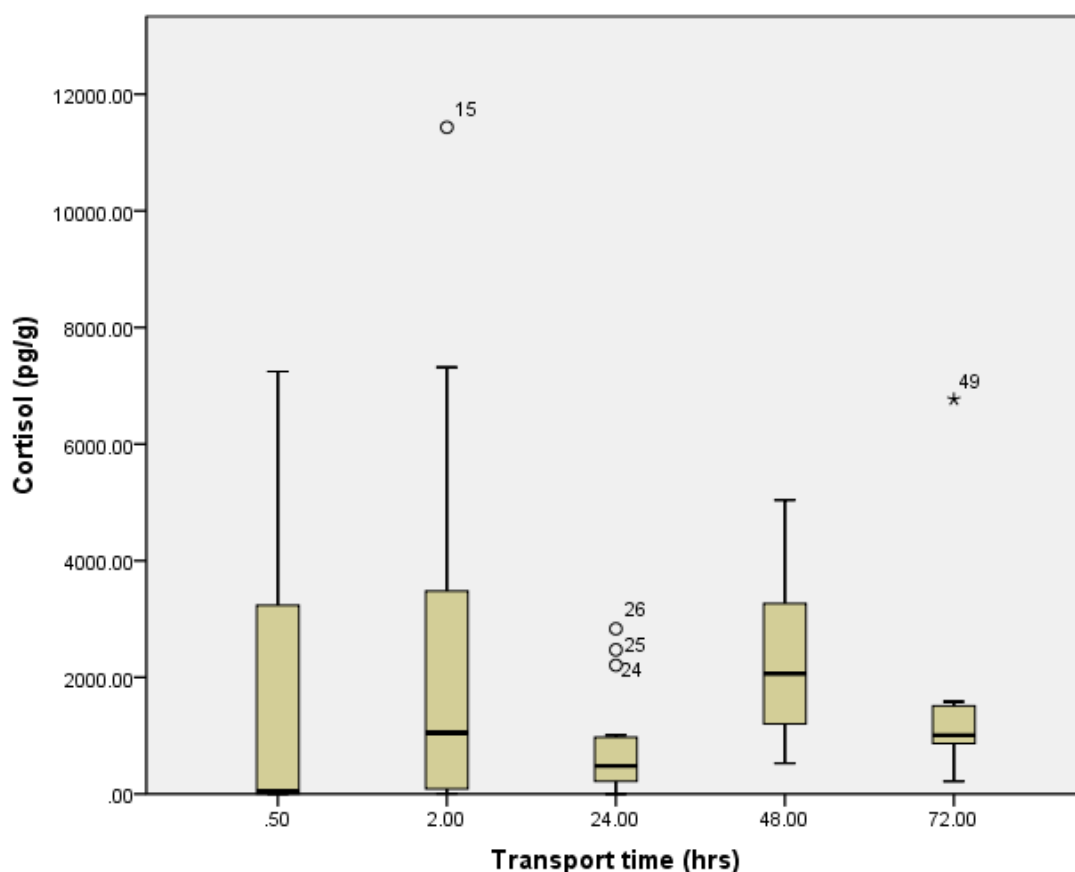


Figure 2.3. Water cortisol concentration (pg/L) after transport durations of 0 (30 min acclimation = .50, 2, 24, 48 and 72 h in common clownfish (N = 36).

TAN increased with increasing transport duration ( $H = 24.0$ ,  $P < 0.001$ ,  $df = 4$ ; Fig. 2.4) with TAN values at 24 ( $P = 0.015$ ), 48 ( $P = 0.020$ ) and 72 h ( $P < 0.001$ ) greater than 0 h. There was no effect of transport duration on pH ( $P = 0.677$ ) which ranged between 8.0 and 8.2 nor on alkalinity ( $P = 0.828$ ) which ranged between 2.7 and 3.5 meq/L. There was a negative relationship between cortisol and TAN ( $R_s = -0.452$ ,  $P = 0.008$ ,  $N = 36$ ; Fig. 2.5).

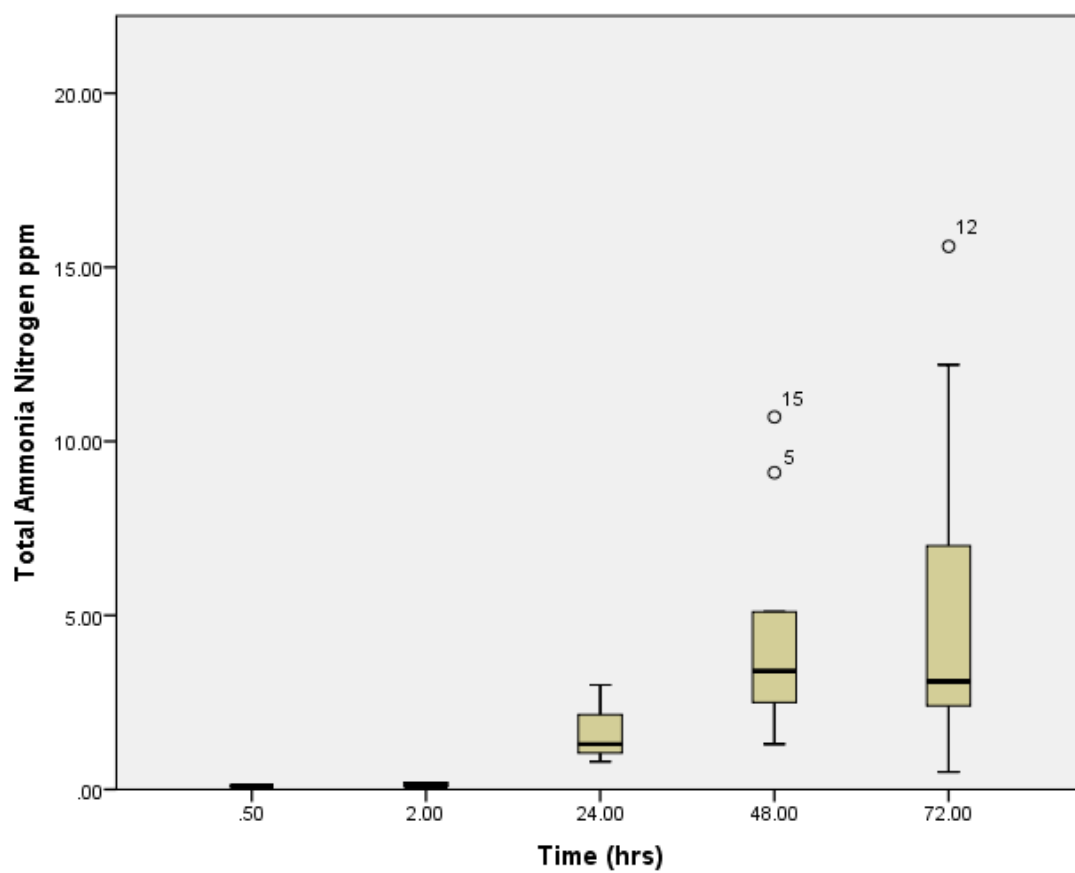


Figure 2.4. Total Ammonia Nitrogen (TAN, ppm) measured after differing transport durations from 0 (30 min acclimation = .50) up to 72 h (N = 36).

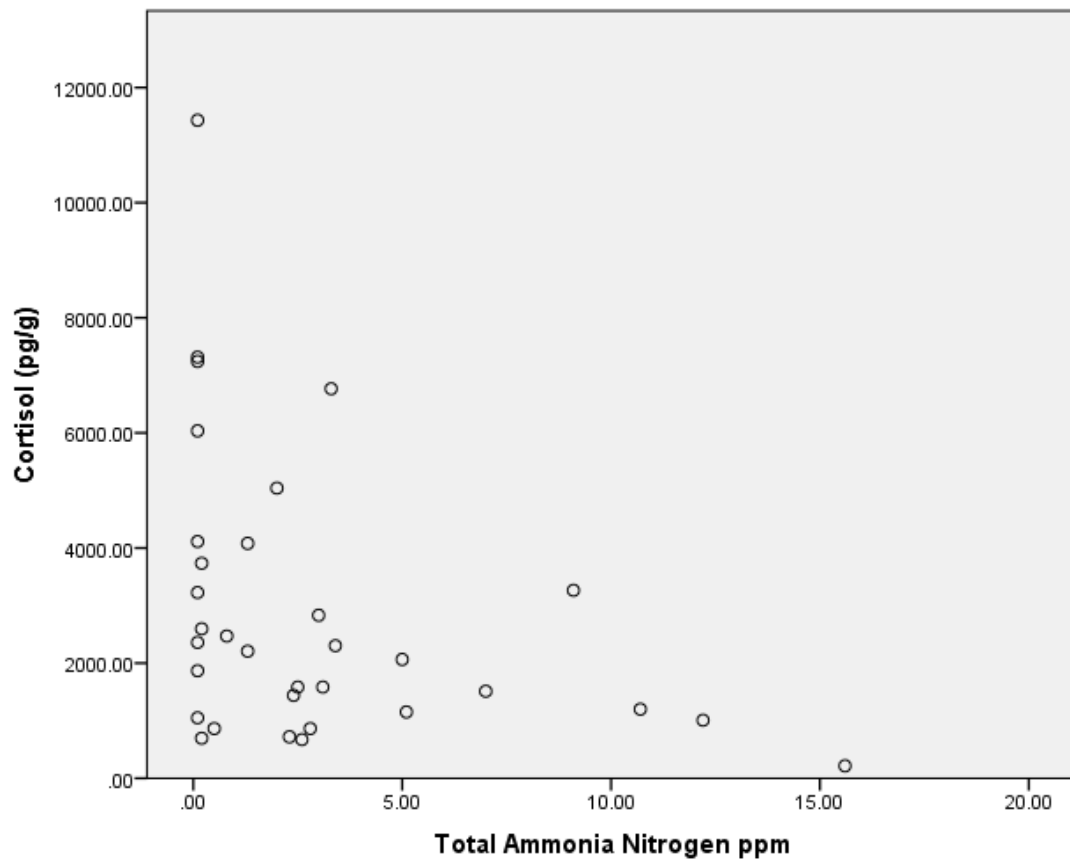


Figure 2.5. The relationship between water cortisol concentrations (pg/L) and total ammonia nitrogen (ppm) (N = 33).

Social behaviours were significantly affected by transport time ( $H = 11.1$ ,  $P = 0.020$ ,  $df = 4$ ; Fig. 2.6) with a dramatic reduction at 48 h ( $P < 0.001$ ) and 72 h ( $P < 0.001$ ) compared with the other treatments. Fish in the 24, 48 and 72 h groups took much longer to resume feeding than those at 0, 2 and 12 h (Table 2.1.;  $X^2 = 40.47$ ,  $P < 0.001$ ,  $df = 2$ ). There was an insignificant trend for social behaviours to decrease with increasing cortisol ( $R_s = -0.645$ ,  $P = 0.062$ ,  $N = 36$ ).

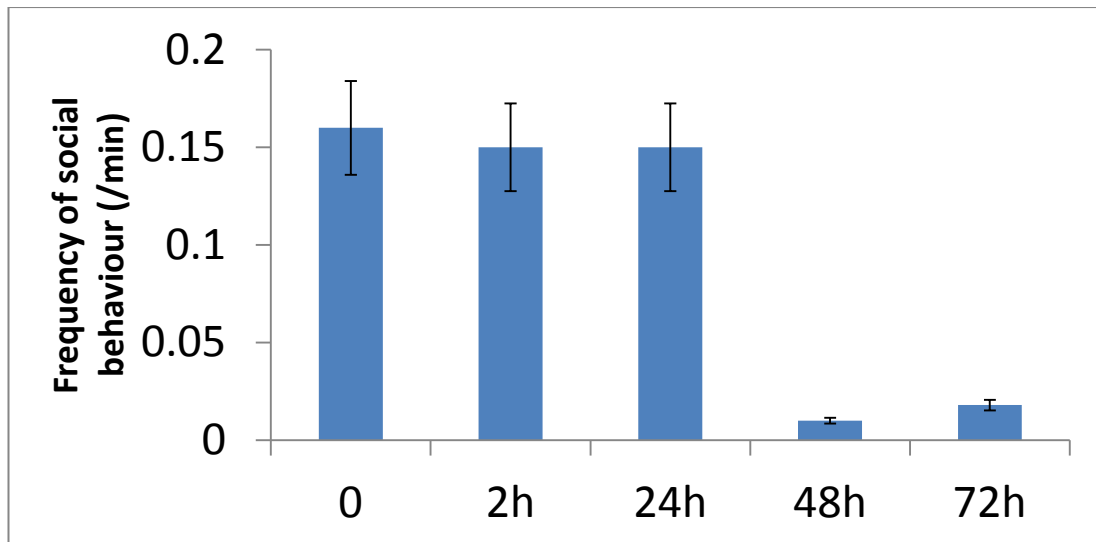


Figure 2.6. Median frequency ( $\pm$ IQR) of social behaviour performed by common clownfish after simulated transport of 0, 2, 24, 48 and 72 h (N = 6 per group). Each bar represents the mean  $\pm$  SEM, n = 6.

Table 2.1. The number of fish that fed over two hourly intervals after simulated transport at 0 (30 min acclimation only), 2, 12, 24, 48 and 72 h over the 6 h observation period (P < 0.001; N = 36).

Transport duration (h)	Number of fish feeding after simulated transport			Total
	0-2 h	2-4 h	4-6 h	
<b>0</b>	4	6	6	16
<b>2</b>	3	5	6	12
<b>12</b>	2	5	6	13
<b>24</b>	0	1	6	7
<b>48</b>	0	0	6	6
<b>72</b>	0	1	5	6
<b>Total</b>	<b>9</b>	<b>18</b>	<b>53</b>	<b>80</b>
Expected	26.7	26.7	26.7	$\chi^2 = 40.47$

*Experiment 2: Does improving water quality reduce stress in clownfish during transport?*

When analysing all cortisol values irrespective of transport duration, cortisol concentrations were higher in the Bioballs water samples (Fig. 2.7;  $U = 386.0$ ,  $P = 0.033$ ,  $N = 48$ ). Only at 48 h was there a significantly higher cortisol concentration in the Bioballs treatment groups compared with those that had no filter materials (Fig. 2.8;  $U = 58.0$ ,  $P = 0.041$ ). There was no significant difference in cortisol between each group at each time point when no filter and Bioballs groups were compared to one another (Fig. 2.9;  $U = 10.1$ ,  $P = 0.074$ ).

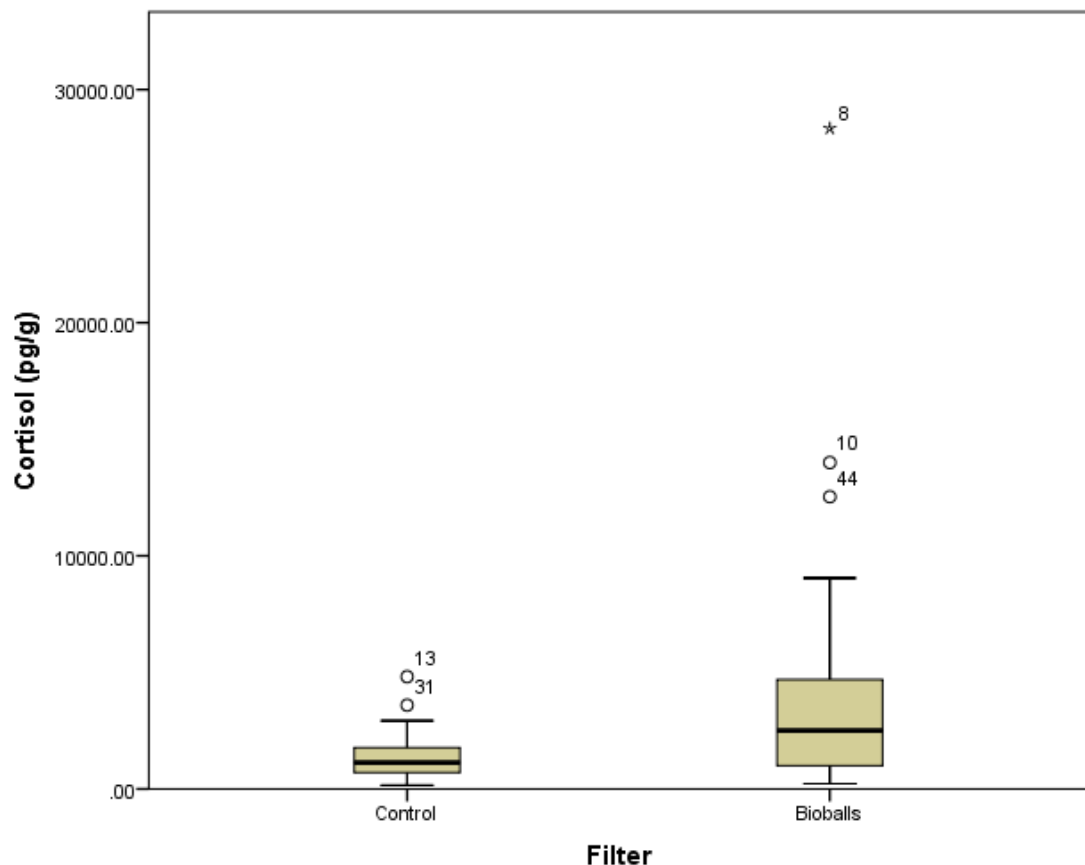


Figure 2.7. Boxplots showing the water cortisol concentration in transported clownfish from 24 to 72h duration when no filter material was present (Control) and when Bioballs filter material was present ( $N = 51$ ).

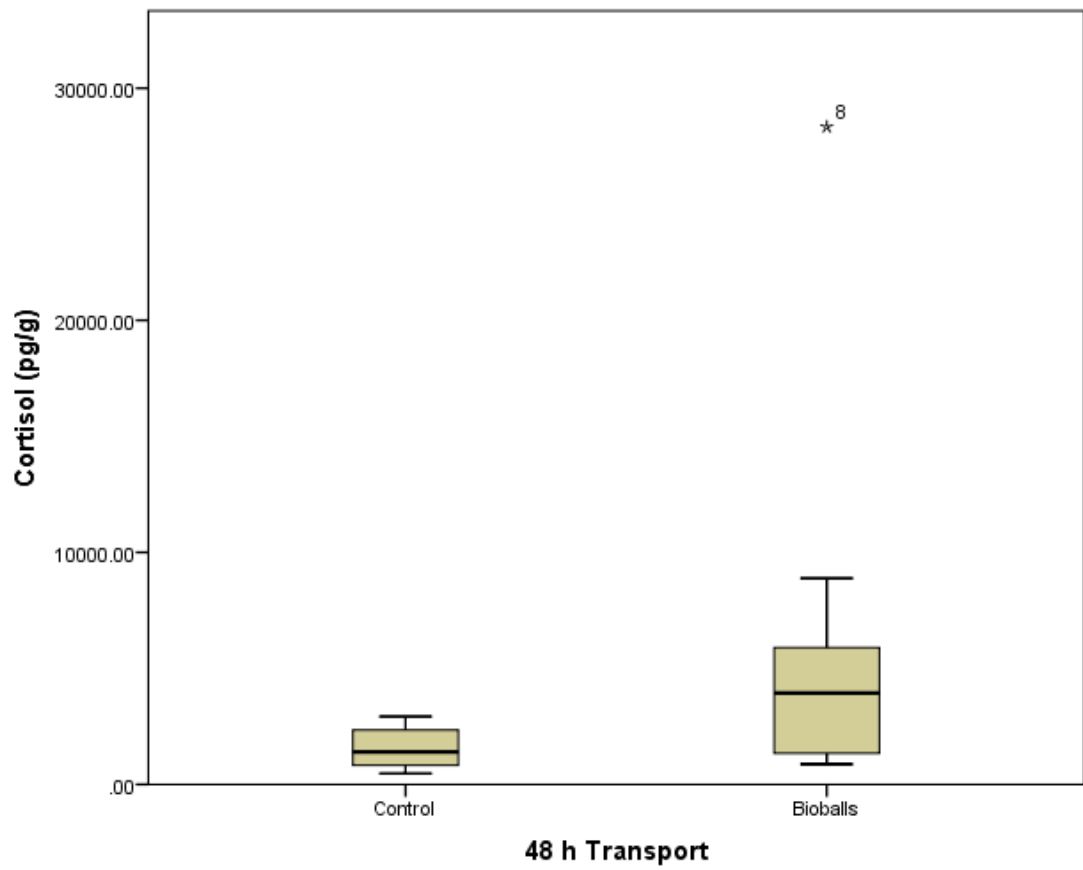


Figure 2.8. Boxplots showing a significant difference between water cortisol measurements after 48 h transport in clownfish with no filter material (Control) and those transported with Bioballs (N = 18).

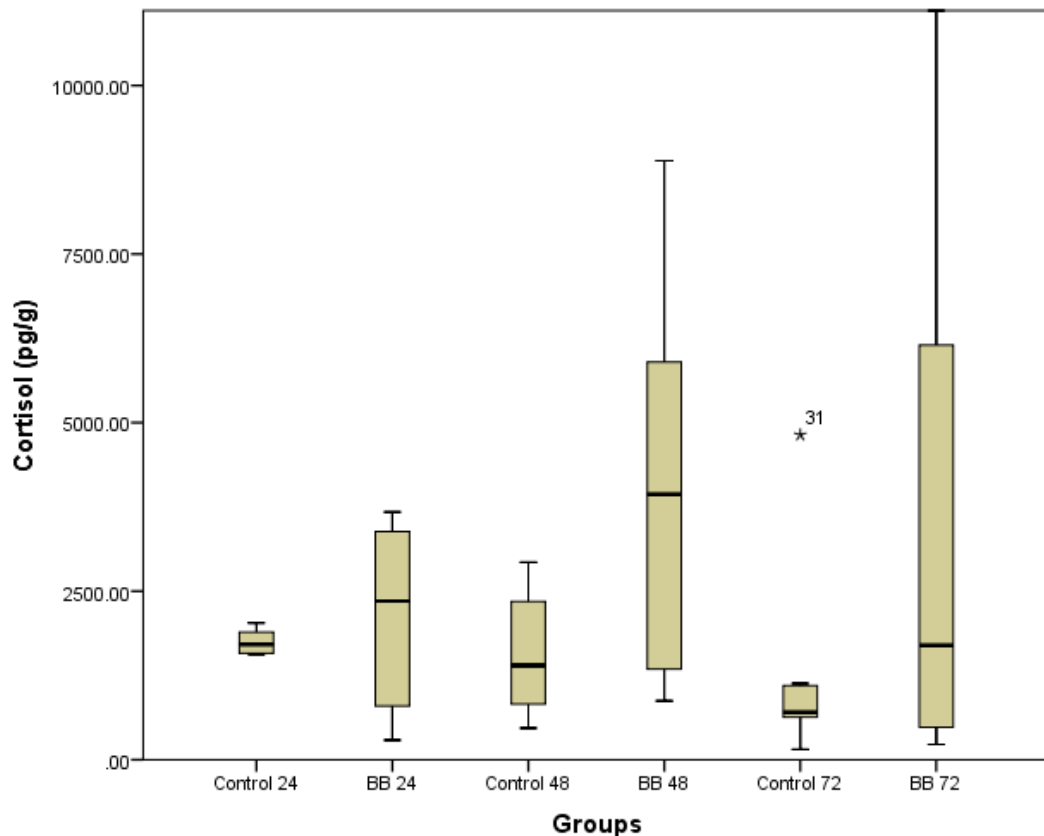


Figure 2.9. Boxplots exhibiting water cortisol concentrations from clownfish transported for 24, 48 or 72 h with no filter media (Control) or Bioballs (BB; N = 51).

As in Experiment 1 nitrite values were below  $<0.1\text{mg/L}$  and often below the limits of detection, therefore, excluded as a significant factor. Total alkalinity ( $P = 0.924$ ) and pH ( $P = 0.141$ ) again were not significantly different over the 24, 48 and 72 h transport durations in the Bioballs transported water samples. TAN exhibited a trend to increase over time when the groups were combined (Control and Bioballs;  $P = 0.076$ ), however, when the Bioballs group was analysed separately there was no significant difference over the three transport durations ( $P = 0.195$ ) and TAN values were substantially lower in the Bioballs groups ( $<3.25\text{ ppm}$ ;  $P < 0.001$  compared with no filter material). In comparison values measured in Experiment 1 ranged up to over 15 ppm.

Social behaviour after Bioballs transport was similar to that seen in those fish that had no filter media at 24 ( $P = 0.945$ ), 48 ( $P = 0.200$ ) and 72 h ( $P = 0.534$ ). As seen in Experiment 1 social behaviour was performed at a higher rate in the 24 h Bioballs



group compared with 48 and 72 h (Fig. 2.10;  $H = 8.63$ ,  $P = 0.013$ ,  $df = 2$ ). Very few fish fed in the Bioballs groups (Table 2.2) which was comparable with the numbers from Experiment 1 at the same time point (Table 2.1). There was no effect of transport duration in the Bioballs treatment on resumption of feeding ( $X^2 = 3.70$ ;  $P < 0.250 > 0.10$ ).

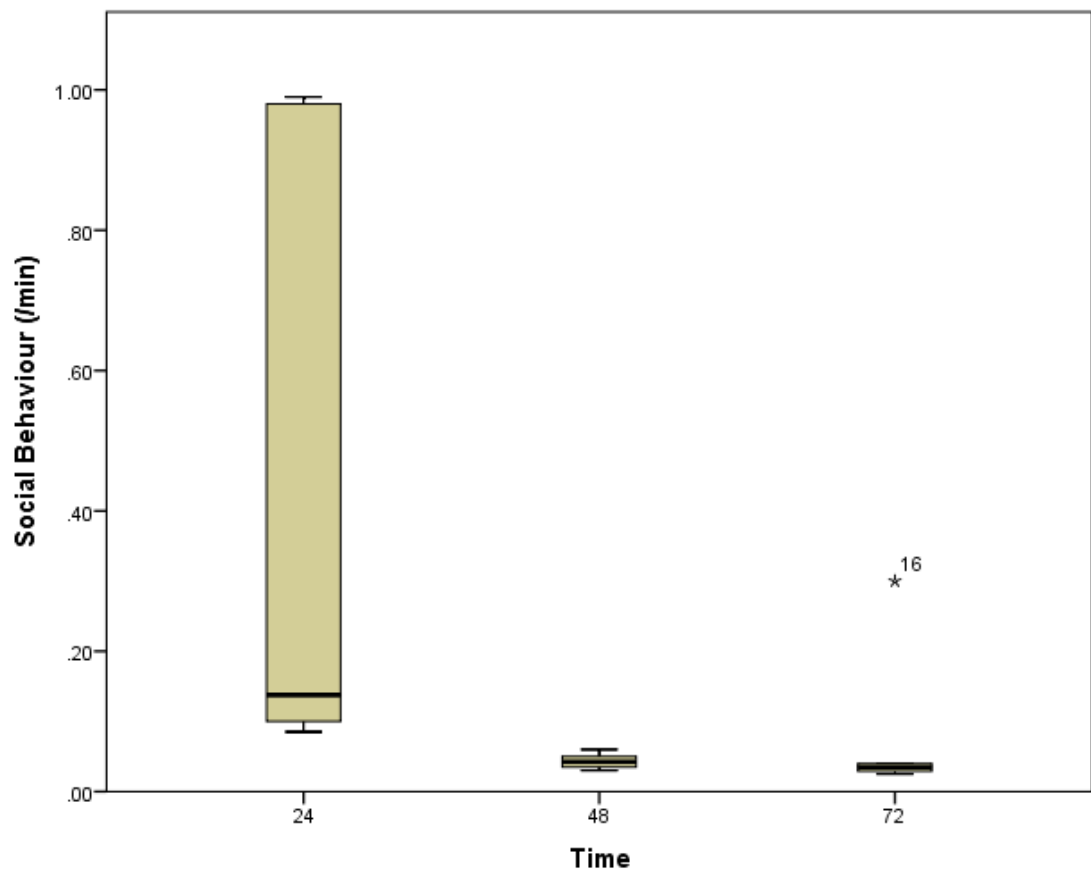


Figure 2.10. Boxplots showing performance of social behaviour in clownfish after simulated transport for 24, 48 and 72 h in the presence of Bioballs (N = 26).

Table 2.2. The number of fish that fed over two hourly intervals after simulated transport at 24, 48 and 72 h over the 6 h observation period in the presence of Bioballs ( $P < 0.25$ ;  $N = 36$ ).

<b>Transport duration (h)</b>	Number of fish feeding after simulated transport			Total
	0-2 h	2-4 h	4-6 h	
<b>24</b>	0	2	6	8
<b>48</b>	1	2	4	7
<b>72</b>	0	3	5	8
<b>Total</b>	<b>1</b>	<b>7</b>	<b>15</b>	<b>23</b>
Expected	7.7	7.7	7.7	$\chi^2 = 3.70$

## Discussion

Longer simulated transport periods resulted in higher cortisol concentrations after 24 h measured as whole body cortisol (24 and 48 h) and as water borne cortisol (48 and 72 h). As this glucocorticoid hormone is the end point of the HPI axis this suggests that transporting common clownfish for more than 24 hours is stressful. Water quality deteriorated as transport duration increased but this was only due to an increase in ammonia (TAN) produced by the fish. Without biological filter material to convert ammonia to nitrite and as part of nitrogen turnover, convert nitrite to nitrate, ammonia concentration would be expected to build up over time. When the Bioballs were present ammonia did not reach such high levels possibly due to the action of the nitrogen cycle. However, the clownfish were still excreting high concentrations of cortisol and in both experiments had delayed recovery from

transport whereby social behaviour and feeding was affected for up to six hours afterwards.

#### *Validation of a non-invasive measure of physiological stress*

For non-invasive measures of cortisol such as the assaying of water-borne quantities of these steroids, there must be a statistically-significant correlation between those found in the water and those within whole body homogenates, blood or plasma of the subjects. There was found to be a clear correlation between water-borne cortisol and whole body homogenates in *A. ocellaris* in the present study. Therefore, it can be inferred that cortisol levels extracted from the water are effectively analogous to those within the whole body samples themselves. Close correlations between water-borne cortisol and plasma cortisol have been noted in various other species, for example the convict cichlid, *Amatitlania nigrofasciata* (Wong, Dykstra, Campbell & Earley, 2008); the sailfin molly, *Poecilia latipinna* (Gabor & Contreras, 2012), European seabass, *Dicentrarchus labrax* (Fanouraki *et al.*, 2008), Atlantic salmon, *Salmo salar* (Ellis *et al.*, 2008) and zebrafish, *Danio rerio* (Félix, Faustino, Cabral, & Oliveira 2013). Exact matches between water-borne and plasma levels of other hormones have also been recorded in the goldfish *Carassius auratus* (Scott & Sorenson, 1994). Thus measuring water cortisol is a viable means of assessing physiological stress in many fish species.

The recovery of cortisol from ‘spiked’ samples yielded interesting results such that incomplete recovery of cortisol was recorded. Recovery rates varied from 34.9% to 99.2%, with a mean recovery rate of 53.8%. However, the higher recovery rates were from cortisol concentrations much higher than measured in the present study. This apparent loss of cortisol using SPE via C18 cartridges suggests that the cortisol levels reported within the present study from water samples may, in fact, be significantly higher than those analysed. This may be due to some interaction with the chemicals that comprise sea water that is not seen or reported in studies of freshwater teleosts (e.g. Scott *et al.* 2008).

According to Diamandis and D’Costa (1988), recovery rates of cortisol and other hormones have been shown to vary depending on the chemical characteristics of the hormone itself. Additionally, it is suggested that the performance of Sep-Pak<sup>®</sup>

cartridges (reflected by the recovery rates from ‘spiked’ samples) may vary between batches of the cartridges. As a result, the authors recommend the application of a Relative Recovery Factor to reflect such incomplete recovery. Nevertheless, the relatively consistent differences between the ‘spiked’ samples and the quantities of hormone recovered meant that the cortisol levels given within the present study, which were those actually recovered recorded, with no correction factor applied provide a reliable measure of relative cortisol release under these conditions. To date, there appears to be a lack of empirical data in the literature outlining the efficacy of cortisol extraction using SPE followed by RIA in salt water compared to fresh water. The results presented in terms of recovery from spiked samples may in this case be anomalous, and perhaps reflective of variations in quality of the batches of cartridges used, or they may be a reflection of relatively low efficiency of hormone recovery from salt water samples. However, further investigation would be required to draw clear conclusions.

#### *The impact of transport on physiological and behavioural stress*

The results of cortisol assays clearly indicated a stress response during transport for more than 24 h. The pattern seen was an increase in whole body cortisol production from 30 minutes to 48 hours, with increasing variation noted between individuals in each group. At 72 hours, whole body cortisol was relatively lower which is potentially linked to increased ammonia at this time point (see Pickering and Pottinger, 2006, in which elevated ammonia is implicated in a suppression of the cortisol response in brown trout, *Salmo trutta*). Certainly there was a negative relationship where high ammonia corresponded to low cortisol in Experiment 1. However, ammonia was not recorded at such high levels when Bioballs were present yet high water cortisol concentrations were detected in these clownfish. This pattern of cortisol production appears to follow that noted for various other fish species subject to long term stress (Pottinger, T.G., personal communication, May 2012). However, there was a delayed response measured from water samples whereby concentrations did increase over time but peaked at 48 and 72 h in experiment 1. This delay or lag in cortisol excretion has been recorded in a number of species such as European seabass, *Dicentrarchus labrax* (Fanouraki *et al.*, 2008), Atlantic salmon, *Salmo salar* (Ellis *et al.*, 2008) and zebrafish, *Danio rerio* (Félix, Faustino, Cabral,

& Oliveira 2013). Exogenous cortisol excreted over the gills may serve a protection function in epithelial integrity and maintaining osmoregulatory homeostasis as demonstrated in zebrafish embryos (Kwong & Perry 2013). However, it may also be the case that physiologically the clownfish were adapting to a stressful situation explaining the decline in cortisol at 72 h whereby homeostasis was achieved (Peter, 2011).

It can be concluded that transport techniques appear to invoke a physiological stress response in *A. ocellaris* that also resulted in a suspension of feeding and reduced social contact at the 24, 48 and 72 h time points. Anorexia is a classic symptom of stress in many fish species (e.g. Hoglund, Gjoen, Pottinger, & Overli, 2007) and increased stress appears to reduce social behaviour in a variety of animal groups including fish (review in Hostetler & Ryabinin 2013). Indeed in *A. ocellaris* a recent study has shown that cortisol concentrations reflect social status (Iwata *et al.*, 2012). There was a nearly significant correlation between cortisol and social behaviours where high cortisol related to low social interaction ( $P = 0.07$ ) providing evidence that stress reduces social behaviour in clownfish but perhaps sample size needs to be increased to obtain statistical significance. Thus it is intuitive that clownfish exposed to a longer stressor will recover more slowly resulting in impaired feeding and interactive behaviours. The reduction of activity can be considered as the individual conserving energy to restore homeostatic balance or there may be a causal relationship between the HPI glucocorticoid release and behaviour that should be explored further in future studies.

The results of the present study validate both water cortisol, resumption of feeding and social behaviour as non-invasive measures of the stress response and clearly indicated that longer simulated transport times caused an elevated release of cortisol and a more prolonged behavioural recovery. Therefore, transport durations over 24 hours should be avoided in common clownfish and these findings may inform the regulations with respect to fish transport. It would be useful given the diversity of fish to test transport stress in other common ornamental species. Further research into the effects of fish size, sex and social grouping may also be necessary to determine the effects of these parameters on whole body and water-borne cortisol.

Many of the water quality parameters did not differ in the transported groups so pH, total alkalinity and nitrite were relatively stable and in the case of nitrite at very low concentrations. Therefore, in the current study these parameters (Harmon 2009) can be excluded from the factors contributing to the stress of the animals. Ammonia (TAN) clearly increased with transport time in Experiment 1 due to waste production of the fish in a confined space. This was also the case in a study where zebrafish were subjected to transport stress, however, this study only recorded ammonia after 72 h (Dhanasiri, Fernandes & Kiron, 2013). The highest recorded ammonia reading in the present study was 15.6ppm, which represents a considerable threat to the health of the fish (At the pH of 7.97 and temperature of 24°C, this equates to 0.739ppm unionised ammonia, NH<sub>3</sub> which is toxic). Ammonia is known to elicit a stress response in a variety of teleosts, for example common carp, *Cyprinus carpio* (Diricx *et al.*, 2013), pacu, *Piaractus mesopotamicus* (de Abreu, Esteves and Urbinati, 2012), goldfish (Sinha *et al.* 2012), Senegalese sole, *Solea senegalensis* (Weber *et al.* 2012) and the yellow catfish *Pelteobagrus fulvidraco* (Zhang *et al.*, 2011). As previously discussed there was a negative correlation between cortisol and ammonia but this disappeared when Bioballs were present and indeed the biological filter material appeared to ameliorate the accumulation of ammonia in Experiment 2 where values were substantially lower. This means adding biological filter material to the transport bags did improve water quality, however, the fish still exhibited behavioural and physiological signs of stress regardless of this. Similar results were found when transporting zebrafish for 72 h where although adding filter bacteria in liquid form reduced ammonia concentrations there was no difference in whole body cortisol in the improved water quality groups compared with the controls (Dhanasiri *et al.*, 2013). The results suggest that the stressor in transport is actually the confinement to a small bag rather than deteriorating water quality. Indeed the Bioballs themselves were large and took up space which may explain why cortisol values were higher in fish with Bioballs present at 48h compared with those that did not since these items would reduce the available space for the fish even further representing greater confinement. There may be benefits of adding filter material (as suggested by Harmon, 2009) but less bulky items could be explored in future studies since reducing ammonia during transport is likely to have health

benefits. Additionally other ornamental fish may respond differently to transport stress thus it would be important to explore filter media utility in their transport.

## **Conclusions**

Measuring water cortisol alongside resumption of feeding and engagement in social interactions were viable alternatives to invasive physiological sampling to assess the stress response in clownfish. The present study demonstrated that transport durations above 24 h were particularly stressful and therefore fish transport should be limited to below 24 h to improve the health and welfare of fish. Although filter material addition did contain ammonia accumulation, clownfish still exhibited a stress response after 24 h. Future studies should explore alternatives to improving water quality and how confinement stress can be reduced on lengthy journeys.

## **Chapter Three: The Impact of Stocking Density and Environmental Enrichment on the Stress Response in *Garra rufa* used in Beauty Treatments**

### **Introduction**

The cyprinid fish, *Garra rufa* (Heckel, 1843), originates from a variety of freshwater habitats (from fast-flowing rivers to turbid ponds) from Southern Turkey to Northern Syria, and from the Tigris, Euphrates and Jordan River basins (FishBase, n.d.). These fish have traditionally been utilised in Turkish bathing establishments, employed for their habit of apparently ‘grazing’ on the skin of bathers. It has been suggested that the activities of *G. rufa* may provide health benefits, for example for those suffering from psoriasis (see Atkins, 2007). From the mid-2000s, *Garra* ‘fish spas’ became very popular in many countries, promoted as a beauty treatment as well as purported ‘therapy’, with a surge in popularity around 2011-12. Clients essentially immerse their whole body in a swimming pool or simply place hands or feet in a fish tank to allow the *G. rufa* to give them a “manicure” or “pedicure” by feeding on dead skin. However, in the UK, a number of health scares in the popular press effectively created an almost overnight decline in the demand for fish spas, following a report from the non-departmental public body (NDPB) The Health Protection Agency (2011). The report highlighted low (but nevertheless possible) risks for the transmission of zoonotic bacterial infections such as mycobacteriosis from fish to human, and also for the transmission of blood-borne viruses such as HIV from human to human through contaminated water and / or contact with fish previously exposed to an infected human client. Indeed a range of pathogenic bacteria associated with *G. rufa* has been identified (Verner-Jeffreys *et al.*, 2012). Similarly, health concerns have led to fish spas being outlawed in several states of the USA, including California in 2013 (California Board of Barbering and Cosmetology, n.d.). Nevertheless, fish spas continue to be used on a worldwide basis in spite of the apparent health concerns for human clients. In addition to potential human health issues, the use of fish such as *G. rufa* in such beauty treatments raises several welfare concerns for the animals themselves.



*Potential welfare issues associated with 'fish spas'*

Firstly, *Garra* are *aufwuchs* (from the German for 'surface growth') feeders, and therefore naturally graze on the film of microfauna and algae occurring on the substrate of their habitat. Yalçın-Özdlieç and Ekmekçi (2006) examined the gut contents of *G. rufa* inhabiting the Asi Basin in Turkey, revealing that the fish's diet is primarily composed of benthic algae, in addition to rotifers and protozoans. The fish may not, therefore, be provided with adequate nutrition in the captive environment, with the commonly-held assumption that the fish 'eat' human skin cells being highly questionable. To date, there is no evidence that *Garra* are able to assimilate human cells, and it is possible that the keratinised epidermal cells of human skin are in fact indigestible to the fish.

Secondly, the design of 'fish spa' systems is typically sparse, with few (if any) furnishings which could be considered as environmental enrichment. *Garra* species originate from diverse environments with often complex three-dimensional topography and features, and the barren nature of the typical spa system may present a welfare issue if the behavioural needs of the animal cannot be fulfilled. Several studies have demonstrated that many fish species actively seek out 'enriched' areas (or conversely, avoid barren environments; review in Sneddon, 2011). In this context, 'enrichment' may be more subtle than merely providing furnishings to vaguely approximate natural features such as plants and rocks, and highly species-specific behaviours may be apparent. Some ingenious studies have highlighted these subtleties. For example, Sessa *et al.* (2008) demonstrated that zebrafish (*Danio rerio*) preferentially spawn in the shallow areas of specially-designed tanks which provide a depth gradient. This mimics natural conditions (where spawning occurs in the shallows), and conferred benefits in terms of offspring survival. Similarly, Chen, Hong, Su and Zhang (2008) demonstrated that great blue-spotted mudskippers (*Boleophthalmus pectinirostris*) exhibit specific environmental preferences, in terms of substrate, temperature and salinity. Furthermore, there is evidence which suggests that the presence or absence of enrichment has an influence on the stress status of certain fish species. For example, Näslund *et al.* (2013) suggest that the baseline cortisol levels of Atlantic salmon (*Salmo salar*) maintained in barren rearing tanks is higher than those reared in enriched systems. Presumably, a lack of enrichment

initiates a stress response in those species and could potentially be considered as a chronic stressor, potentially leading to the subsequent widely-documented effects including elevated cortisol resulting in immunosuppression, impaired growth and reduced fecundity.

Thirdly, fish spas tend to be relatively densely-stocked with fish to ensure a suitable experience for the client. The Ornamental Aquatic Trade Association (2008) recommends a stocking density of 2.5 kg/m<sup>3</sup> for freshwater fish species however, fish spas generally stock their tanks in excess of these recommendations which can be detrimental to the welfare of the fish (Wildgoose, 2012; BIAZA Aquarium Working Group, personal communication, 2012). This presents further welfare concerns for several reasons. Crowding may be a direct stress factor for the fish concerned, although the responses of species can vary greatly in response to particular stocking densities and the effects of stocking density are complex. Whilst some species, for example gilthead sea bream *Sparus aurata*, respond negatively to increased stocking density (Montero *et al.*, 1999), many shoaling species actively seek out conspecifics. Very high stocking densities may even reduce aggression in some species, for example Malawi cichlids. It is well-known amongst aquarists maintaining these species that heavily-stocked systems experience fewer aggressive interactions between individuals, although the highly aggressive and territorial natures of such fish are the reasoning behind such husbandry practices (personal observation). In view of the variability of effect regarding stocking density, it seems reasonable to suggest that each species of fish has an optimal stocking density (or range of densities) which limits the stress experienced by each individual. An additional issue with high stocking densities is that infectious diseases may be easily transmitted between individuals. Fish coming into more frequent contact with each other in more densely-stocked systems present a greater risk of transferring pathogens to one another. Whilst many commercially-available fish spa systems feature ultraviolet sterilizer (UV) units, the effectiveness of these relies on regular maintenance from the user, and UV sterilizers will not, in any case, completely eradicate the possibility of pathogens affecting fish in any aquarium system (Dr. Peter Burgess, personal communication, February 2010). The higher biomass within densely-stocked systems also presents issues of water quality in closed systems and can result in elevated ammonia and nitrite concentrations that are toxic to fish,

causing stress, disease and mortality (Mitchell & Tully, 2009). Water quality is, of course, a major concern when maintaining fish. Maintaining optimal water quality requires efficient mechanical and biological filtration to respectively trap suspended solid waste for subsequent removal, and ensure the effective microbial conversion of ammonia (the primary waste product of the fish themselves) to nitrate, via nitrite. Both ammonia (particularly in its unionised form,  $\text{NH}_3$ ) and nitrite ( $\text{NO}_2^-$ ) are extremely toxic to fish and these compounds are of particular concern, although long-term exposure to nitrate ( $\text{NO}_3^-$ ) is also well-known to have deleterious effects (Camargo, Alonso & Salamanca, 2005). The filtration units on commercially-available fish spas may often be well-designed and fundamentally able to adequately maintain water quality within ideal limits providing adequate maintenance is performed, and that filters have been matured to provide adequate microbial populations to process nitrogenous wastes. However, establishing a mature biofilter is an involved process, with the necessary microbial populations often taking several weeks or even months to reach sufficient levels to process the waste produced by a tank of fish (depending on stocking densities). If live fish are added to systems featuring newly-established biofilters, then spikes of ammonia and nitrite will be expected due to inadequate populations of Ammonia-Oxidising Bacteria (AOBs) and Nitrite-Oxidising Bacteria (NOBs) within the biofilter. Such elevated levels of ammonia and nitrite may seriously compromise welfare, and in many cases lead to the death of the fish. Thus, holding *G. rufa* at unnaturally high densities over the recommendations for ornamental fish may reduce their welfare.

The aim of this study was to determine if reduced stocking density and provision of enrichment reduced stress in *G. rufa* by non-invasively measuring water cortisol. The impact of stocking density on water quality was also monitored to explore whether stress was correlated with altered pH, ammonia, nitrite and nitrate. A further aim was to test whether feeding on human hands altered cortisol excretion and may be considered as a potential stressor. The objective of the present study was to provide novel information on the welfare of *G. rufa* used in spa treatments, and relate this to their husbandry. This may inform guidelines of animal welfare and government regulatory bodies to improve the health of individuals in the fish spa industry, if welfare is compromised by lack of enrichment or high stocking densities.

## Methods

The present study took place prior to and during the negative publicity in 2011/12 and received ethical approval from the University of Chester.

### *Animal husbandry*

*Garra rufa* fish ( $n = 300$ ;  $1.28 \pm 0.45\text{g}$ ) were imported from a farm in Singapore by Maidenhead Aquatics, U.K. and transported to the Animal Management Centre, (Reaseheath College, Cheshire, U.K.) where they were housed in a commercial aquarium system (CASCO Europe Ltd., U.K.) in six tanks (40 x 37 x 30 cm). Excess stock were housed in a larger tank (120 x 37 x 30 cm) tank during experiments. Freshwater was filtered using Tetra IN 300-100 Plus, (Melle, Germany) internal filters and the temperature maintained at  $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$  by Tetra Tetrattec<sup>®</sup> HT50, (Melle, Germany) heaters. Fish were held under 12:12 light/dark photoperiod and fed Tetra TabiMin<sup>®</sup> sinking pellets twice daily (3% of bodyweight) as recommended for cyprinids (Shafi, 2007). All fish were acclimated for eight weeks at a stocking density of  $2.5 \text{ kg/m}^3$  (OATA, 2008) prior to experiments commencing.

### *Experimental design*

Fish were assigned at random to one of four treatment groups (Table 3.1) where each group had either optimal stocking density and either barren (OP/B) or enriched (OP/E) conditions ( $n = 10$  fish per tank,  $n = 6$  replicate groups) or alternatively overstocked density with either barren (OS/B) or enriched (OS/B) conditions ( $n = 15$  fish per tank;  $n = 6$  replicate groups). Once placed in these groups fish were allowed to acclimate under the conditions describe above for seven days prior to experimentation commencing.

Table 3.1 Treatment groups showing stocking density and provision of enrichment using *Garra rufa* fish (n = 6 groups per treatment).

	Code	Stocking Density	Enrichment	Number of <i>Garra rufa</i> per group
1	OP/B	Optimum	Barren	10
2	OP/E	Optimum	Enriched with plastic aquarium grade plants	10
3	OS/B	Overstocked	Barren	15
4	OS/E	Overstocked	Enriched with plastic aquarium grade plants	15

#### *Assessment of water cortisol concentrations pre- and post-manicure treatment*

To investigate if feeding on human hands was stressful water samples were collected prior to and after hands were inserted into tanks containing the *G. rufa* groups (Table 3.1). The filtration and flow into all tanks was turned off 30 minutes prior to collection of the first water sample and fish were not fed that day until the experiments has ended to ensure these factors did not interfere with water cortisol concentrations. Samples were taken at the same time each day to prevent diurnal fluctuation in cortisol. To prevent the samples being contaminated with human skin derived cortisol and other external skin derived contaminants latex gloves were worn at all times by the researcher. To obtain normal baseline concentrations of cortisol 1 litre of water was removed carefully from each tank into sterile containers and frozen at -20°C. Subsequently human volunteers placed their hands into each tank for a 15 minute period to simulate a fish spa manicure, allowing the *G. rufa* to feed on any dead skin. A second water sample was collected 30 minutes afterwards. The human volunteers had their hands inspected to exclude those with any infections or open wounds; hands and forearms up to elbow were washed vigorously using 10 cm<sup>3</sup> HiBiSCRUB® a disinfecting hand-wash. Subsequent rinsing with copious amounts of water removed any residues. To control for any cortisol which may have come

from the volunteers hands water samples were taken from tanks which had no fish in prior to insertion of hands and after hands as described above (n = 4).

At the end of each water sampling period the fish were weighed by netting and placed into pre-weighed beakers containing 500 ml of aquarium water (to 0.01g; Ohaus Pioneer Precision PA512C balance). Each fish was returned to its tank and the wet calculated by subtracting the beaker weight from the fish plus beaker weight. At the end of the experiment the fish were added to Reaseheath College's Zoological Collection as exhibit fish.

### ***Filtration and solid phase extraction***

Filtration and water cortisol measurements were made at the University of Chester in Dr Robert Coleman's laboratory who uses a validated Enzyme Linked Immunosorbent Assay (ELISA; Hughes et al. 2010) for cortisol for reasons of convenience and time. Each water sample was defrosted at room temperature ( $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ) over the 24 hour period and filtered using a diaphragm pump (Flowgen Vacuubrand, Germany) through filter paper (0.45  $\mu\text{m}$ , Sartorius Stedim Biotech, Germany) to remove debris. Filtered water was passed through the solid phase extraction cartridges (Sep-Pak<sup>®</sup> Plus C18, Waters Ltd., U.K.) after they were charged with 5ml methanol at a rate of c. 20 ml min<sup>-1</sup> using a peristaltic pump (Gilson<sup>®</sup> Miniplus 3, U.S.A.). The filtered water was collected and frozen at -20 for later analysis of pH, total ammonia nitrogen (TAN) and nitrite as described in Chapter 3.

### ***Solvent extraction***

The cortisol was eluted by pipetting 4 ml of ethyl acetate through the cartridge and into a clean test tube. Elutions were evaporated using a heating module (Pierce Reacti-Therm<sup>™</sup> Model 18780, U.S.A.) in a fume cupboard by blowing a slow stream of nitrogen gas onto the elutions whilst warming to a temperature of 45°C, as described by Ellis, James, Stewart and Scott (2004). The residue was re-constituted with 500  $\mu\text{l}$  of a 0.1% buffer solution using 98% minimum bovine serum derived albumin (Sigma-Aldrich<sup>®</sup>, U.S.A.) dissolved in phosphate buffered saline (PBS; 5.42 g/l  $\text{NaH}_2\text{PO}_4\text{H}_2\text{O}$ , 8.66 g/l anhydrous  $\text{Na}_2\text{HPO}_4$ , 8.7 g/l  $\text{NaCl}$  and 9 ml distilled  $\text{H}_2\text{O}$ ).

### ***Cortisol determination***

Enzyme Linked Immunosorbent Assay (ELISA) was employed to determine cortisol concentration. Plates containing hydrophilic microtiter wells (Maxisorp™, Nunc, Germany) coated with 50 µl of cortisol antibody (R4866) diluted at 1:12,000 with PBS solution at pH 7.0 were incubated for 24 hours at 4°C after which any unbound conjugate was washed off with a solution of PBS and Tween® 20 (Sigma-Aldrich®, U.S.A.). 50 µl of the re-constituted residue and 50 µl of cortisol horseradish peroxidase steroid conjugate (HRP) was added to the wells. Plates were sealed and incubated in the dark at room temperature ( $21^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ) for three hours. The plates were re-washed and sealed to prevent the HRP dehydrating.

100 µl of Enzyme Immunoassay (EIA) substrate solution was added to each well (solution comprises 0.05 M at pH 4.0 of EIA citrate buffer comprising 4.80 g/l anhydrous citric acid and 10 ml distilled water, 40 mM of EIA ABTS [2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diamonium salt] comprising 0.329 g/l ABTS and 15 ml distilled water, 0.5 M of EIA H<sub>2</sub>O<sub>2</sub> at 2.0% comprising 500 µl of 8M H<sub>2</sub>O<sub>2</sub> and 7.5 ml distilled water). The plates were sealed and shaken until a colouration developed of approximately 1.00 optical density. The absorbance was read using a Dynatec MR 500 plate reader. Known standards were run from 50 to 1000 pg of cortisol to create a standard curve from which the cortisol concentration of unknown samples could be calculated. To give a concentration per g the cortisol data was divided by the total wet weight of fish obtained. The blank samples of water taken from the tanks which contained no fish was analysed for cortisol which was under the limits of detection. Similarly the samples taken from empty tanks after volunteers had placed hands in had cortisol concentrations from zero to <50 pg/l, thus it was unlikely human cortisol was a significant factor.

To validate the assay procedure, recovery of standards, specificity of the assay and inter-assay variation was explored. Recovery of standards was at the 99% level (equation for recovery of 0-500pg standards  $y = 0.9955x + 54.484$ ,  $r^2 = 0.9917$ ); the assay specificity was high when comparing recovery between standards and *G. rufa* samples ( $F_{1,22} = 0.12$ ,  $P = 0.73$ ). There appeared to be no inter-assay variation in the samples; however, only two plates were used.

## Statistical Analysis

Data for water cortisol concentrations for each treatment group, before and after insertion of hands, were normally distributed (Shapiro-Wilk's tests for normality: OP/B,  $P = 0.731$ ; OP/E,  $P = 0.769$ ; OS/B,  $P = 0.065$ ; OS/E,  $P = 0.913$ ). Therefore a Two-way ANOVA was employed to determine whether enrichment or stocking density affected cortisol, pH, TAN, nitrite and nitrate for all the data and also separately for the data before hands and after hands. Paired T-tests analysed whether there was a significant change in the variables before and after hands. All tests were conducted using SPSS (Version 19) software and two-tailed tests were employed throughout.

## Results

When the cortisol data was pooled there was a significant effect of stocking density where overstocked fish were excreting higher cortisol compared with optimally stocked ( $F_{1,44} = 17.6$ ,  $P < 0.001$ ; Fig. 3.1) but no influence of enrichment ( $F_{1,44} = 1.6$ ,  $P = 0.205$ ) nor an interaction between the two factors ( $F_{1,44} = 0.237$ ,  $P = 0.629$ ). When analysing cortisol values measured prior to hands stocking density affected cortisol in a similar manner ( $F_{1,20} = 11.2$ ,  $P = 0.003$ , Fig. 3.2) but there was no effect of enrichment ( $F_{1,20} = 1.0$ ,  $P = 0.324$ ), however there was an interaction ( $F_{1,20} = 8.746$ ,  $P = 0.008$ ; Fig. 3.3). Post hoc tests demonstrated that only OS/B differed from the other treatments (OP/B  $P = 0.004$ ; OP/E  $P = 0.025$ ; OS/E  $P = 0.012$ ). There was an influence of both stocking density ( $F_{1,20} = 38.4$ ,  $P < 0.001$ ) and enrichment ( $F_{1,20} = 7.2$ ,  $P = 0.014$ ) on cortisol measured after hands but no interaction ( $F_{1,20} = 0.1$ ,  $P = 0.792$ ). Cortisol was relatively greater in the overstocked tanks compared with optimally stocked and higher in enriched tanks compared with barren conditions (Fig. 3.4).



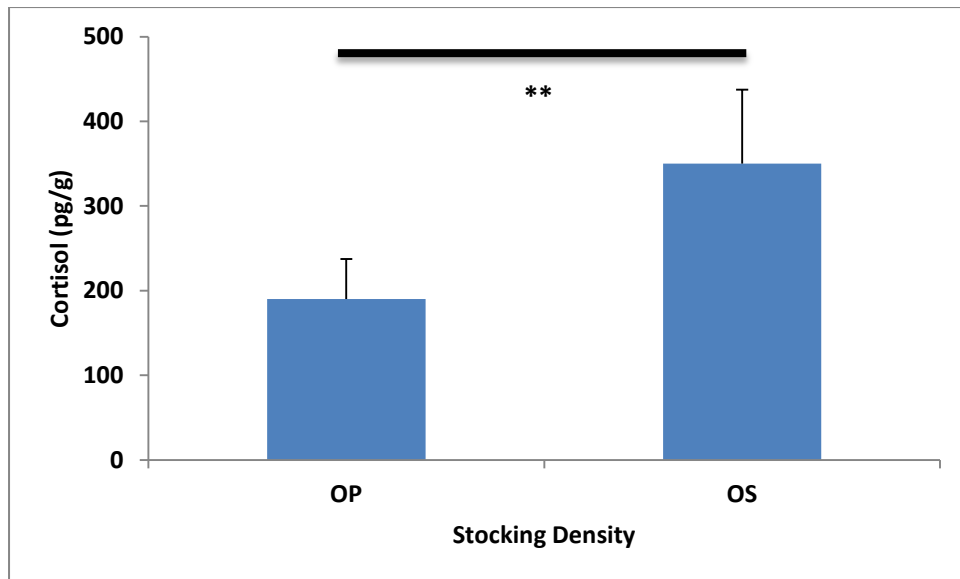


Figure 3.1. Mean cortisol (+se) in water collected from tanks containing *Garra rufa* fish that were optimally stocked (OP) or overstocked (OS; n = 12 tanks per group; \*\*P<0.001).

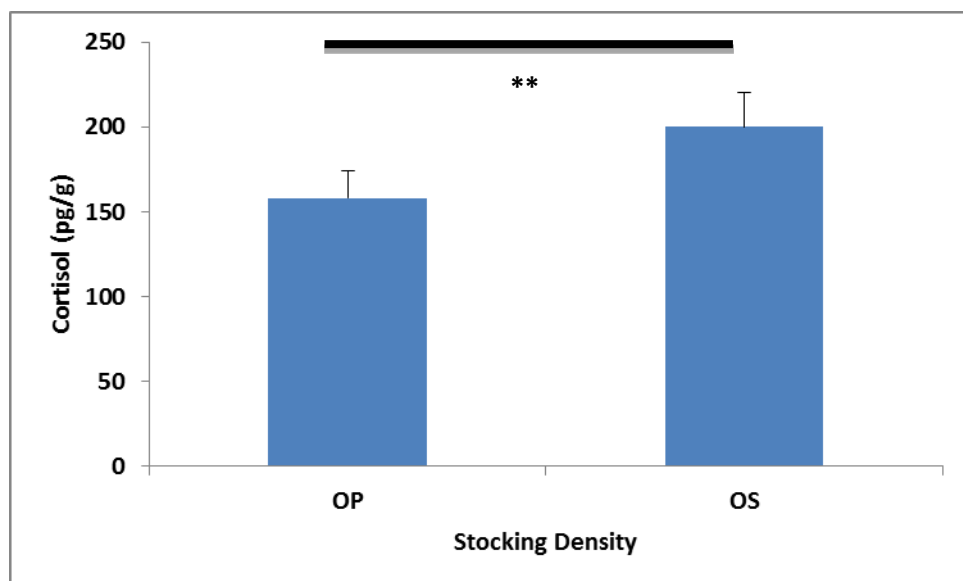


Figure 3.2. Mean cortisol (+se) in water collected from tanks containing *Garra rufa* fish that were optimally stocked (OP) or overstocked (OS) before insertion of human hands into the tanks (n = 6 tanks per group; \*\*P = 0.003).

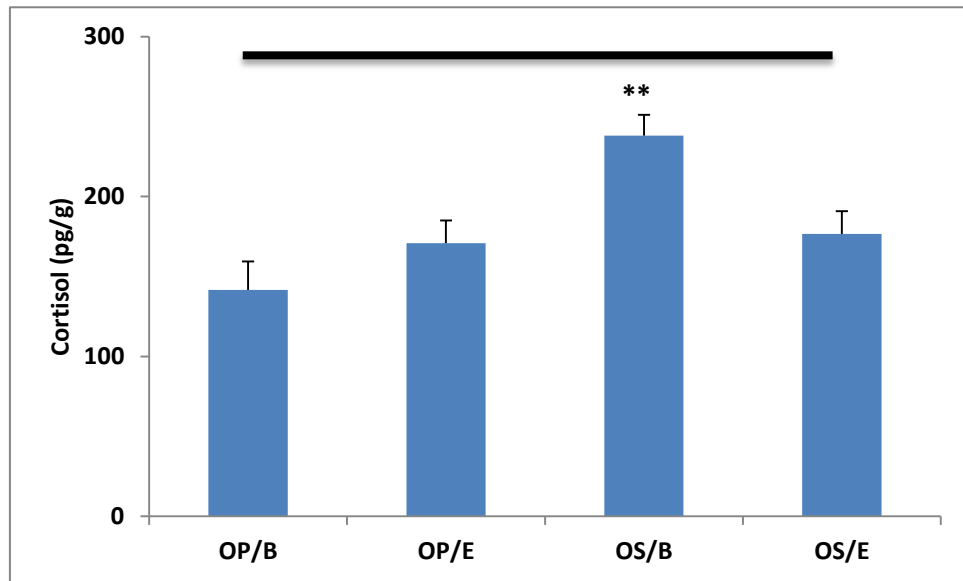


Figure 3.3. Mean cortisol (+se) in water collected from tanks containing *Garra rufa* fish that were optimally stocked (OP) or overstocked (OS) in barren (B) or enriched (E) conditions before insertion of human hands into the tanks (n = 6 tanks per group; \*\*P = 0.008; OS/B differed to all other groups).

Table 3.2. Mean cortisol concentration with standard error before and after presentation of hands in tanks containing *Garra rufa* fish held under optimally stocked (OP) or overstocked (OS) density and under barren (B) or enriched (E) conditions (n = 6 tank per treatment). T test analysis is also shown with significant P values in bold.

Treatment	Before Mean (se)	After Mean (se)	T Value	P Value
OP/B	141.6 (21.2)	162.8 (12.3)	-1.208	0.281
OP/E	170.7 (17.2)	256.8 (50.4)	-2.965	<b>0.031</b>
OS/B	236.0 (15.5)	393.6 (29.5)	-5.374	<b>0.003</b>
OS/E	176.6 (17.0)	508.3 (74.2)	-5.047	<b>0.004</b>

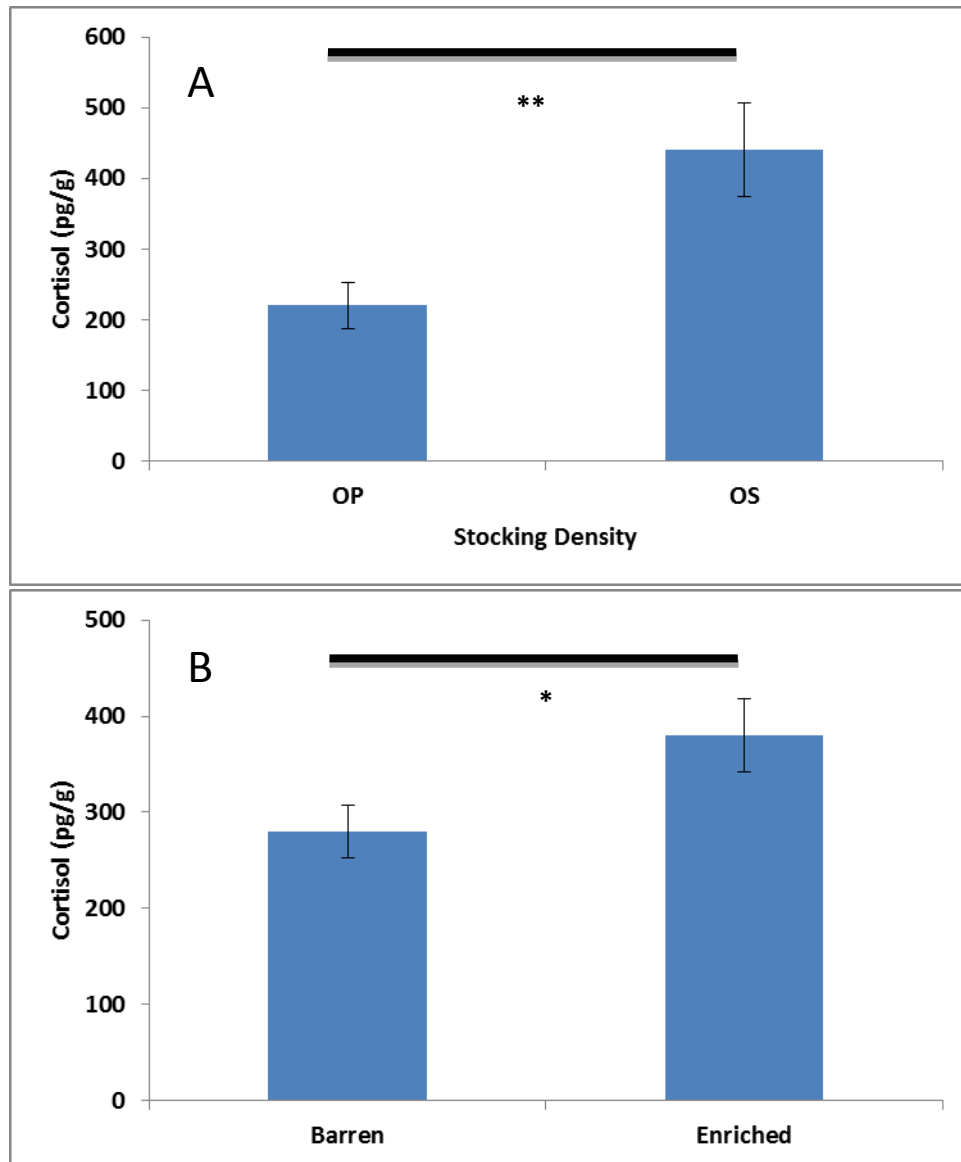


Figure 3.4. Mean cortisol (+se) in water collected from tanks containing *Garra rufa* fish that were (A) optimally stocked (OP) or overstocked (OS) or (B) held under barren or enriched conditions after insertion of human hands into the tanks (n = 6 tanks per group; \*\*P < 0.001; \*P = 0.018).

Placing hands in the tanks had no effect on cortisol concentrations measured in OP/B (T = -1.2, P = 0.281; df = 5) but significantly increased cortisol in all other treatment groups (OP/E T = -2.9, P = 0.031; OS/B T = -5.374, P = 0.003; OS/E T = -5.0, P = 0.004; Table 3.2).

TAN and Nitrite concentrations were extremely low (< 0.01 mg/l) and often below the limits of detection so both were excluded as significant factors. pH was not affected by either stocking density (P = 0.562) nor enrichment (P = 0.366) and was measured in a narrow range 7.05 – 7.20.

## Discussion

Stocking *G. rufa* above recommended densities resulted in relatively higher cortisol production at baseline prior to presentation of hands for the fish to feed upon and also after hands were inserted. Environmental enrichment did affect stress whereby fish held under overstocked barren conditions had higher cortisol concentrations compared with the other groups. After feeding upon human hands enriched fish produced more cortisol than fish held under barren conditions which may be possibly due to a combination of being overstocked and the addition of enrichment actually reduces the available space for the fish to use resulting in confinement stress. Exposure to human hands did elevate cortisol concentrations in three groups (OP/E, OS/B and OS/E) suggesting that competition for feeding may have been stressful or the positive reward of feeding was responsible for the increase.

### *Impact of stocking density*

The influence of stocking density on fish health is complicated with contradictory results obtained both intra- and inter-specifically. For example, in rainbow trout, *O. mykiss*, cortisol was elevated at low stocking densities but not at high density even though brain serotonergic activity indicated chronic stress (Laursen, Silva, Larsen & Höglund 2013). In contrast Larsen, Scov, McKenzie and Jokumsen (2012) found that high stocking density resulted in higher cortisol production compared with low density in this species. Pickering and Pottinger (1987) also found that cortisol increased with increasing stocking densities in rainbow trout, yet other studies on rainbow trout have found that cortisol decreases with increased stocking density (Procarione, Barry & Malison, 1999). A recent study by McKenzie *et al.* (2012) found no effect of stocking density on cortisol concentrations in rainbow trout. Thus the impact of stocking density appears to be context specific to each type of study and set up. The results of the present study also suggest an interesting context-specific relationship between the use of barren tanks and provision of enrichment (see Fig. 3.3); elevated cortisol was enriched tanks under optimal stocking compared to barren, whereas the trend is reversed in more heavily-stocked systems. In *G. rufa* the higher stocking density resulted in higher water cortisol concentrations when the

data was pooled and before and after hands were presented to the fish. Here, the conditions that these fish are kept in spas were simulated to allow the results to have relevance to this industry, therefore, high stocking densities should be avoided in *G. rufa* in the fish spa context.

One can speculate on the mechanisms or explanations that cause high cortisol in the high density groups. For example, increased numbers of fish result in higher competition and possibly agonistic social interactions for feeding opportunities. In juvenile turbot, *Scophthalmus maximus*, high stocking densities resulted in increased aggressive interactions over food but only when that food source was defensible (Li, Liu & Blancheton, 2013). In the present study, the human hand can be considered as a defensible resource in contrast to scatter feeding which disperses widely and cannot be defended. A study on brown trout, *Salmo trutta*, demonstrated that defending a food source was more stressful than playing the role of intruder sneakily obtaining food (Kaspersson, Höjesjö & Pedersen, 2010) thus *G. rufa* at higher stocking densities may have to engage in competitive interactions more often than in lower densities. Alternatively, there may be more food available per fish in low stocking densities, therefore, less or even no agonistic behaviour in the lower density groups. The impact of defensible food resources and competition on stress in *G. rufa* should be explored in future studies.

Increased densities of fish often place a biological burden on the filtration system and as such results in higher waste products due to the inability of the filter to cope with the increased ammonia production (Camargo, Alonso & Salamanca, 2005; Mitchell & Tully 2009). However, in the present study water quality parameters were not significantly affected by the experimental paradigm and were well within optimal ranges for many ornamental freshwater teleosts. Therefore, water quality parameters can be excluded as a causal factor in the present study. This is possibly due to the use of a semi closed system where clean freshwater was continuously added to the system and the efficiency of the filtration system used. If much higher densities of *G. rufa* were used then perhaps water quality would deteriorate so future studies should investigate whether much higher stocking densities affect water quality.

### *Impact of environmental enrichment*

Contrary to expectations providing enrichment did not reduce stress measured as cortisol excreted into tank water. When the data were pooled and before hands were presented there was no effect of enrichment but after feeding upon human hands there was higher cortisol concentrations recorded in the overstocked group. One possible explanation is that the provision of structural enrichment actually reduced the amount of space available to the fish, therefore, when hands were placed into the tank this reduced space and resulted in a confinement stress. Confinement is a standard stress test in fish that often results in peak plasma cortisol production. Many studies have shown that enrichment has a positive impact upon stress and welfare. For example, in Atlantic salmon provision of enrichment ameliorated the impact of stressors compared with fish held under barren conditions (Näslund *et al.*, 2013). Cyprinids such as common carp, *Cyprinus carpio*, roach, *Rutilus rutilus* and chub *Leuciscus cephalus* and the salmonids rainbow trout, brown trout and Arctic charr, *Salvelinus alpinus*, all demonstrate a profound acute stress response to confinement (Pottinger, 2010). However, the provision of enrichment had no measurable effect on the stress response in zebrafish (Wilkes *et al.*, 2012). Therefore the enrichments employed in this study may not be suitable; the plastic aquarium grade plants and gravel may not reflect the natural habitat of *G. rufa*. Okur and Yalçin-Özdilek (2008) recorded the natural composition of *G. rufa* inhabited Turkish mountain streams and described coarse substrates including pebbles and organic detritus. In Lake Kinneret, Israel, *G. rufa* feed largely on rocky habitats (Goren & Ortal, 1999). Therefore, larger pieces of rock or pebbles may be more suitable enrichment for *G. rufa* if studies wish to simulate their natural habitats. Additionally these fish were only acclimated to the plastic plants and gravel for seven days prior to experimentation and may require a longer acclimation period for any beneficial effect of enrichment to be seen. However from the results of the present study it would be prudent to refrain from recommending the provision of enrichment to *G. rufa* tanks until further testing is conducted.

### *Are fish affected by feeding on hands?*

In three out of four treatment groups cortisol was elevated after hands were inserted into the tanks. There are two possible explanations; firstly that the insertion of hands was stressful and; secondly that fish were excited to feed on this novel food source and the cortisol values obtained do not reflect a negative stress. As discussed in Chapter 2 fish display anorexia when stressed (Hoglund, Gjoen, Pottinger and Overli, 2007) yet all fish were observed to graze readily on the human hands that were presented to them. Additionally fish in the OP/B treatment did not show an elevated stress response after feeding on hands and two of the other groups had high stocking densities perhaps explaining why there was an elevation (OS/E and OS/B). In the group OP/E where stocking density was optimal it is possible that the enrichment constrained the amount of space available which was further reduced when hands were introduced. In favour of the second hypothesis it is known that animals often increase cortisol in response to rewarding stimuli. For example, Atlantic cod, *Gadus morhua*, increased cortisol in anticipation of a food reward (Nilsson *et al.*, 2012). Thus it may be that these fish are displaying a natural physiological response to the provision of food. Feeding in captivity often results in anticipatory behaviour and physiological release of glucocorticoids (e.g. François' langurs, *Trachypithecus francoisi*; Krishnamurthy, 1994; capuchins, *Cebus apella*; Ulyan *et al.*, 2006; and bears, leopards, and pumas; Carlstead, 1998). This does not necessarily mean this is negative, however, the fact that cortisol is relatively higher before feeding in overstocked groups does suggest stocking density does influence stress and welfare of these fish.

### **Conclusions**

High stocking density does appear to elevate stress measured as water cortisol in *G. rufa*, therefore, this result suggest spas should consider stocking these fish at OATA (2008) recommended densities to improve welfare. If fish are held at high densities over a long period this could result in chronic stress reducing immune function and allowing the outbreak of zoonoses such as mycobacteriosis caused by *Mycobacterium* sp. (Verner-Jeffreys *et al.*, 2012) that affect public health. The provision of enrichment did not conclusively improve welfare and indeed when stocking densities were high actually increased stress. Future studies should

investigate more naturalistic enrichment, however, employing enrichment in *G. rufa* spas cannot be recommended based upon the results of the present study. Feeding on human hands did result in elevated cortisol in three treatment groups, however, two of these were overstocked and the other already had enrichment present. Therefore, hands may reduce the availability of space for fish to swim and therefore could represent a confinement stress. Alternatively the high cortisol values may be linked to a positively rewarding experience of feeding. Future studies should explore stocking density, different types of enrichment and the use of larger tanks to ameliorate the reduction of space in the experimental context.



## Chapter 4: General Discussion

The findings of both studies in Chapter Two and Chapter Three have shown that physiological stress can be measured non-invasively to gauge how stressful two different experimental paradigms were on ornamental fish. In the first experiment, the impact of transport on the hypothalamic-pituitary-interrenal (HPI) axis which elicits cortisol release was measured in the water containing the common clownfish to determine whether placing fish in standard transport conditions resulted in HPI activity. Clearly transport durations of 24 h or more did exert a challenge to homeostasis as measured by excretion of cortisol into the transport bag water. This was validated by whole body cortisol concentrations and also the time taken for the fish to recover behaviourally after transport. Again behavioural recording is non-invasive and demonstrated that fish exhibited a greater latency to resume feeding and reduced social behaviours when subject to transport over 24 h. Cortisol was measured in water containing groups of *Garra rufa* fish in optimally stocked or overstocked densities in either barren or enriched tanks. The results indicated that overstocking did result in relatively higher concentrations of cortisol but the influence of enrichment on the stress response was less clear. Enrichment had no effect but appeared to increase stress when human hands were presented to the fish possibly reflecting a confinement stress due to reduced space for the fish to use when hands were also inserted into the tank. Therefore, the non-invasive approaches adopted within these studies were useful indicators of the physiological state of the animal and may reflect how these practices impact upon the welfare of the fish. These non-invasive measurements of stress may also be valuable in studies of other aquatic animals such as amphibians (Gabor, Bosch, Fries & Davis, 2013) or other species of fish (e.g. Friesen, Chapman & Aubin-Horth, 2012) since physiological sampling is often a challenge to welfare and can involve terminal sampling whereby the animal is killed to obtain a blood or whole body sample.

### *Transport of ornamental fish*

There were a number of limitations in the study in Chapter Two. The simulated transport aimed to be as realistic as possible in its replication of commonly-used

transport techniques within the aquatics industry. However, in many cases, parameters such as temperature may fluctuate considerably, and these may have an effect on the stress and survival rates of the fish (see Chow, Chen & Teo, 1994). Nevertheless, manipulating temperature to reflect such real life scenarios was deemed to be outside the scope of the study, and the transport containers were maintained at  $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ . Future studies should seek to address whether a loss of temperature is an additive stressor in the transport of ornamental fish.

The nature of fish transport dictates that there will be inevitable disturbance to the container in which the fish is placed, due to packing / unpacking, movement of vehicles, etc. This disturbance may present additional stressors to the animals (and as such, were not included in the present study since only transport duration was investigated; Chapter Two). However, effectively replicating such conditions in a realistic manner, and in a controlled and repeatable fashion, presents numerous challenges. It may be possible, now the water cortisol measures have been validated in clownfish, to take water samples from fish which have imported by overseas suppliers and measure both water cortisol and behavioural recovery. Obviously this would need to be implemented in a wide range of users of ornamental fish such as pet shops and public aquaria as the variety of species imported, stocking density as well as transport distance and duration vary considerably but it may be a first step in understand the stress of transport in a more realistic setting.

A number of interesting potential further areas of study can be identified to provide a more comprehensive understanding of the impact of transport on ornamental fish. These include the following: The use of alternative species and investigating the response according to the same transport protocols. Interspecific variations in the responses to transport are widely accepted by experienced aquarists (personal observation). Identifying species which are known to ship with relatively few problems, and comparing their response to more delicate species, would perhaps generate interesting data. Another avenue would be the use of very low dose anaesthetics, and their influence on water quality as well as fish stress and health. It has been suggested that freshwater fish mortality may be reduced via the use of anaesthetics such as MS-222, clove oil and benzocaine (Pramod *et al.*, 2010), with less deterioration in water quality as a result of reduced activity, and perhaps lower

stress levels. However, many anaesthetics can cause a stress response in fish (review in Sneddon, 2012) thus this would need to be tested with caution. Further investigation into the effects of water quality and the influence of additives to transport water would be useful. For example, products which render ammonia relatively harmless (such as Kordon AmQuel<sup>®</sup>) or preparations which are claimed to reduce fish stress levels (such as API Stress Coat<sup>®</sup>) may be of interest. However in Chapter Two, only one type of filter media was tested and was successful in reducing ammonia but this did not reduce stress. Therefore, improving water quality might not have such a great impact as increasing the amount of space available to transported fish thus increasing the transport bag size and amount of water may be a viable means of increasing welfare. This is likely to meet resistance in the industry since increased weight equates to increased shipping costs. Yet users of ornamental fish may be willing to pay more if their stock arrives in better condition and in greater health. Thus the findings of Chapter Two are relevant to improving fish welfare during transport such as ensuring transport times are less than 24 h and future studies could provide novel data on other aspects of transport that could make the practice less stressful for the fish.

#### *The use of Garra rufa fish in fish spas*

The high stocking densities seen in *G. rufa* spas appear to be stressful for the fish and, therefore, the results of Chapter Three will be useful in informing regulations with regard stocking density in these establishments. The standard barren tanks which are the norm for *G. rufa* tanks, however, do not result in stress and conversely the provision of enrichment in these aquaria cannot be recommended since it would seem that this actually increases cortisol production when fish fed on human hands. The trade in *G. rufa* exploded in the UK in 2011 with numerous fish spas appearing in a relatively short space of time and 23,000 to 35,000 fish imported on a weekly basis (Stinton, 2012). However, after the negative publicity on the health risk they posed only a few hundred are imported per week with trade severely affected (Stinton, 2012). Thus, although the findings of Chapter Three are useful as we know very little about this species of fish, the applicability may have less impact for the industry. However, these spas are still common in Europe and in other geographical locations. Future studies may explore other factors such as interspecific interactions

and if *G. rufa* form dominance hierarchies where subordinates are subject to chronic social stress as seen in other species of captive fish (e.g. salmonids - see Gilmour, DiBattista & Thomas, 2005; Sneddon, Schmidt, Fang and Cossins, 2011). Although hands and feet of clients are rinsed prior to immersion into the fish tanks, often moisturiser, fake tan and nail varnish are used by clients and these may contain chemicals toxic to the fish. Thus, whether residues are actually removed by rinsing and whether fish ingest and are affected by these substances would be a very interesting avenue to pursue. Obviously understanding what causes stress in the fish spa environment and reducing the stress of *G. rufa* is of paramount importance since stressed fish are more prone to infection and any disturbance to long term homeostasis can lead to the outbreak of disease. Welfare notwithstanding, such animal health concerns are of financial importance to salons operating *Garra* spas, as fish losses and the subsequent replacement of stock represent a not inconsiderable outlay. Zoonotic organisms such as *Mycobacterium* species which fish are known to have naturally, and whose pathogenicity increases with stressed, immunosuppressed hosts, could also pose a serious health risk to the public if the fish are feeding on skin with open wounds. Thus, improving the fish spa to improve the health and welfare of *G. rufa* is key if these salons continue to operate.

#### *Improvements in fish welfare*

Russell and Burch (1959) were instrumental in instigating the ethos of the '3Rs' into experimental research; namely reduction, replacement and refinement. Reduction refers to the reduction in the numbers of animal used in experimentation; refinement is improved methods to reduce the impact on animal welfare and replacement is the use of alternatives to protected animals (such as cephalopods and non-embryonic vertebrates). Adoption of non-invasive approaches in physiological sampling is a vital refinement to improve experimental animal welfare since normally invasive techniques involve restraining or catching the fish, anaesthetising and causing tissue damage to obtain a blood sample or for small fish the amount of blood needed results in terminal sampling (very small species, or juveniles of larger species, have so little blood that researchers have no choice but to kill the fish and use whole body measures). Thus measuring cortisol in the water refines the manner in which stress can be measured. Adoption of this approach for relevant aquatic species would improve the welfare of captive animals. Other hormones from water samples have

also been validated (e.g. estradiol in African cichlid fish *Pseudocrenilabrus multicolor victoriae*, Friesen et al. 2013; testosterone, 17 beta-estradiol, progesterone, prostaglandin F(2 alpha) and 11-ketotestosterone, Kidd, Kidd & Hofmann, 2010) thus hormone analysis may no longer require invasive approaches for aquatic species. Another useful advantage water sampling has in terms of the 3Rs is that multiple samples can be taken from the same individual, thus reducing the number of animals used in experiments where time series data is required. With invasive sampling, physiological measures taken over a range of time points would require at least five animals per time point whereas using holding tank water does mean only five animals could be used, and water serially sampled over time. This approach may also assist in controlling interspecific variation often seen in the stress response of fish (e.g. Thomson, Watts, Pottinger & Sneddon, 2011) as well as allowing within individual responses to be tracked during, for example, recovery after imposition of a stressor. Overall measurement of such responses represents a major step forward in humane experimentation.

Future refinements of measuring cortisol in holding tank water are needed since siphoning water from the tanks or placing fish into small chambers to collect cortisol may be stressful. Thus the development of a sensor or filter that can be placed in the water where the fish remains relatively undisturbed would be a substantial improvement. Polar Organic Chemical Integrative Samplers (POCIS) are used to detect various chemicals in water bodies. For example, these samplers have been used to measure pesticides and metabolites in the field (Ibrahim, Togola & Gonzalez, 2013) and androgenic-like and estrogenic-like compounds in wastewater treatment run off (Jálová *et al.*, 2013). Thus these POCIS samplers could be employed in experimental and public aquaria to measure a range of biochemicals water-soluble organic chemicals are passively absorbed from water. These samplers can be left for many months in a water body (Alvarez *et al.*, 2007) and have been employed in studies investigating estrogenic effects on fish in natural rivers (Vermeirssen *et al.*, 2005). POCIS would require validation for cortisol collection, however this is a viable route to reduce any impact of water collection on the fish. Another possible avenue for future investigation is the development of a biosensor that can measure cortisol in a small water sample based upon a dip stick system or by applying water to a sensor similar to those used in pregnancy test kits. Indeed a salivary cortisol

sensor has been developed for human saliva by Arya, Chornokur, Venugopal and Bhansali (2010), where cortisol concentrations are provided within 40 minutes of sampling. The sensor analyses untreated saliva for cortisol could replace tests such as radioimmunoassays, enzyme-linked immunosorbent assays or liquid chromatography, all of which require time and pre-treatment of saliva. Such a biosensor would provide a rapid means of measuring cortisol and possible stress thus allowing carers or researchers to intervene to alleviate stress within the hour rather than some time later. The ease of use of such a biosensor might promote adoption by not only researchers but also by the ornamental industry including public aquaria and aquaculture where fish welfare is essential to the economic return of the industry.

### *Conclusion*

This thesis has validated the use of non-invasive measures of stress in two differing contexts to address questions in fish welfare. Measurement of cortisol using holding water is a valid means of determining the impact of transport and stocking density. OATA guidelines allow the transport of fish for up to 72 hours but it does appear that transport for 24 hours or more is stressful affecting subsequent behavioural recovery in common clownfish. Thus transport durations should possibly be reduced to a maximum of 24 hours. Future studies should investigate disturbance during transport, the use of beneficial chemicals for the fish or for water quality and temperature fluctuation as possible factors in reducing fish welfare during transport. High stocking density as seen in fish spas did lead to relatively higher stress in *G. rufa* fish. Therefore, these spas should stock at recommended lower densities to improve the welfare of this species. Providing enrichment did not have a beneficial effect on *G. rufa* measured as water cortisol, therefore, enrichment cannot be recommended. Future studies should explore social interactions and the cleanliness of client's hand and feet as possible stressors in fish spas since the health of these fish is paramount to prevent zoonotic disease transmission. These studies have provided a route to refining physiological sampling in fish by using a non-invasive technique as well as possibly reducing the numbers of fish used in future physiological experiments if this approach was adopted by other researchers in the field.

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## Appendix 1

Table A1.1 Ethogram for *Amphiprion ocellaris* showing all behaviours measured in a pilot study. A preliminary analysis demonstrated only social behaviour ( $P = 0.015$ ) and feeding ( $P = 0.022$ ) differed between 0 h and 48 h.

<b><u>Name</u></b>	<b><u>Description of behaviour</u></b>
Wobble	Fish is stationary and sways and rolls from side to side while the pectoral fins beat unsynchronised.
Head bobbing	Fish is predominantly stationary but raises its anterior end then lowers it while raising its posterior end; this may be repeated.
Shimmy	Two fish lie parallel to each other and shake or 'shudder' simultaneously.
Resting	Fish rests on the substrate using its pelvic fins for support. Fish may also tilt onto its side.
Gulping	Fish opens mouth fully as if gulping water inwards, often combined with erecting all fins and lowering of the buccal cavity.
Surface	Fish rises to the surface for a gulp of air, as fish descends bubbles may exit the operculum. Alternatively, fish may not gulp air, but its dorsal fin breaks the surface.
Flashing	Fish turns its side to face an object such as a rock and quickly rubs its flank against it.
Hiding	Fish remains close to an object (no more than 3cm away) and may hide either inside, behind or under it.
Hug	Fish swims extremely close to an object directly hugging it, (no more than 3cm away).
Attack	Fish swims directly towards another fish at speed followed by a bite within no more than one second.
Chase	One fish pursues a second fish for more than one second.
Evade	Fish flees in response to being chased or attacked by a conspecific.
Fighting	Two fish face off biting at each other's face or flanks, holding onto the pectoral fins in an aggressive interaction.

	The fins may be erected during fighting.
Picky	Fish picks at objects and the substrate as if foraging.
Shudder	Fish repeatedly shudders rapidly along the body. The fish may also direct this behaviour towards another fish, generally while turning side on to the other fish and rolling slightly.
Upright tilt	Fish raises anterior end so the body is positioned at approximately a 45 degree angle. The fish then shudders. This may be carried out by smaller individuals as a submissive behaviour.
Submission	Upon being challenged by a conspecific, the fish lowers its anterior end, when possible it descends further within the tank or swims away from the other fish.
Stationary	Fish stops movement and remains in the same position within open water. Fins are erect, the pectoral and caudal being used to maintain position. Fish may also undulate to help maintain position.
Swimming	Fish is moving around in open water with fins erect and appears not to be displaying any other behaviour.
<b>Social behaviour</b>	<b>Behaviour displayed when a fish swims in parallel with the possible intent to shoal or interact.</b>
Darting	Fish swims at high speeds with fins held flat against its body, either smoothly or sporadically with quick pauses.
Social Behaviour	Fish swims alongside another fish, both swimming the same pattern. Fish may perform this in small groups which appears to the observer as shoaling. This behaviour can be seen being performed by fish on either side of a piece of dividing glass between two adjacent tanks.
<b>Feeding</b>	<b>Fish obtains and consumes a food item.</b>

## Appendix 2

Recovery of known standards diluted to provide concentrations between 0 and 1200 pg in the sea water used for the experimental treatments (Table A2.1).

Table A2.1: Recovery of cortisol from ‘spiked’ samples showing percentage recovery and overall recovery from the test samples.

<i>pg added</i>	<i>pg recovered</i>	<i>% recovery</i>	<i>Mean % recovery</i>	<i>Overall % recovery</i>
0	13.64615			53.79711
0	21.80535			
0	16.77375			
0	18.27665			
150	62.2818	41.5212	44.20593	
150	57.7479	38.4986		
150	73.2718	48.84787		
150	71.9341	47.95607		
300	104.6381	34.87937	42.23966	
300	108.64	36.21333		
300	159.2294	53.07645		
300	134.3685	44.7895		
600	298.2347	49.70578	51.96297	
600	366.4791	61.07984		
600	310.5536	51.75893		
600	271.844	45.30733		
1200	442.0241	36.83534	76.77986	
1200	997.1133	83.09277		
1200	1190.616	99.21797		
1200	1055.681	87.97338		

### Appendix 3

The relationship between body weight and water cortisol excretion in common clownfish demonstrating no influence of body weight upon cortisol concentration (Fig. A3.1;  $R_s = -0.200$ ,  $p = 0.217$ ,  $n = 36$ ).

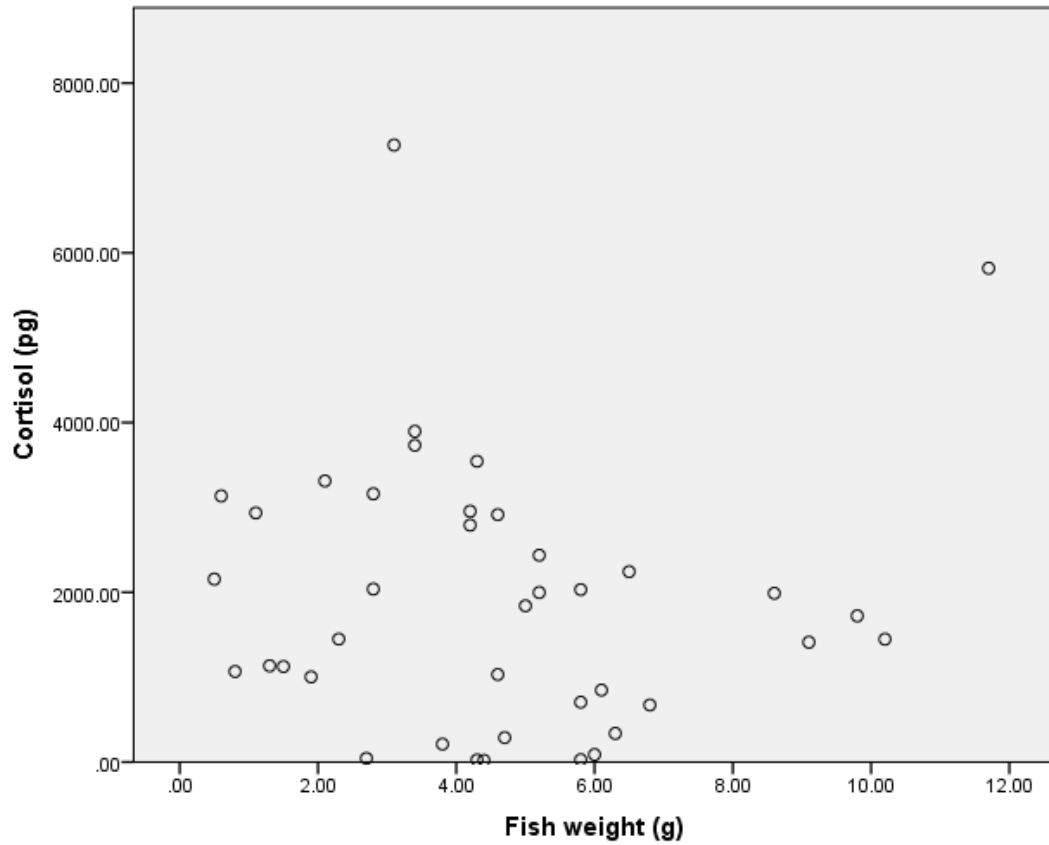


Figure A3.1 The relationship between cortisol (pg/L) and body weight (g) in common clownfish ( $R_s = -0.200$ ,  $p = 0.217$ ,  $n = 36$ ).



#### **Appendix 4: Protocol for Sep-Pak<sup>®</sup> water extractions**

Cortisol was extracted from the water sample onto Solid Phase Extraction (SPE) cartridges according to the following protocol (after T.G. Pottinger, personal communication, 4<sup>th</sup> March 2010). (*Vinyl / latex gloves were worn during all water cortisol extractions to prevent contamination.*):

1. Water was kept frozen, and refrigerated if not used immediately (i.e. within one hour) after defrosting.
2. Water samples were transferred to a tared balance and the weight recorded (= volume of sample to be extracted)
3. Water samples were pre-filtered, using a vacuum flask with 0.45µm nitrocellulose filters.
4. SPE cartridges (Sep-Pak<sup>®</sup> C18 Plus, Waters Ltd) were conditioned and equilibrated as follows: Using two separate 5ml syringes, 5ml methanol (HPLC grade), and then 5ml distilled water, were gently pushed through. Cartridges were not allowed to dry out between methanol conditioning and equilibration with distilled water, to prevent a reduction of the C18 sorbent bed's efficiency, which could result in lower recovery. Cartridges were therefore prepared immediately prior to extraction and not in advance.
5. SPE cartridges were labelled, and connected to apparatus. Water sample was forced via peristaltic pump through the SPE cartridges at a maximum flow rate of 5ml/min (higher flow rates resulting in lower analyte recoveries).
6. SPE cartridges were removed and washed with 5ml distilled water followed by air to remove as much moisture as possible (in order to prevent splitting of the cartridges during freezing). The cartridges were then frozen at -20°C.

7. The adsorbed steroids were eluted within a vacuum manifold (Waters Ltd) into a 12ml tube with 5ml ethyl acetate.
8. The eluate was dried down under a stream of N<sub>2</sub> (at 40°C) in a heating block, then reconstituted in 0.5ml ethyl acetate, before being stored at -20°C.

## **Appendix 5: Cortisol radioimmunoassay (RIA) procedure**

Following elution from the SPE cartridges, samples were assayed according to the following protocol (after T.G. Pottinger, personal communication, 4<sup>th</sup> March 2010). (*Vinyl / latex gloves were worn during all water cortisol extraction to prevent contamination.*):

### **Preparation of Reagents**

#### *3H cortisol stock solution*

1. (Volpato et al., 2007-3H) vial cortisol (60Ci mmol<sup>-1</sup>, GE Healthcare, TRK 407) was made up to 5.0ml with toluene: ethanol (9:1)
2. The stock solution was stored at -20°C.

#### *3H-cortisol working solution*

1. 12.5ml ethyl acetate was added to a clearly labelled scintillation vial (Snaptwist<sup>®</sup>, PE, 6.5ml, VWR).
2. To this, 10µl of 3H-cortisol was transferred to provide working solution.
3. The solution was mixed well using a vortex mixer.
4. A 50µl aliquot of working solution was transferred to a 5.0ml vial.
5. 5.0ml of scintillation fluid (Ecoscint<sup>®</sup> A, AGTC Bioproducts) was added, and cap tightened.
6. The solution was mixed by inversion, and the vial transferred to scintillation counter. Cortisol was assayed using a 3H protocol, with 12,000-20,000 dpm being acceptable.

7. Counts below acceptable range necessitated the addition of a further aliquot of <sup>3</sup>H cortisol stock solution and recounting; counts above the acceptable range necessitated dilution of the working solution appropriately.
8. Working solution was stored at -20°C.

*Standard inert cortisol*

1. 10mg cortisol (Sigma, H-4001) were weighed out in a glass scintillation vial. 10ml ethanol were added, = 1mg cortisol ml<sup>-1</sup> (**A**)
2. 9.95ml ethyl acetate were added by pipette into a vial, and 50μl **A** added, = 5μg cortisol ml<sup>-1</sup> (**B**).
3. 9.9ml ethyl acetate were added by pipette into a vial, and 100μl **B** added, = 50ng cortisol ml<sup>-1</sup> (**C**).
4. The above working standard solution was stored at -20°C.
5. 7 glass scintillation vials were labelled **D-J**.
6. 4.2ml ethyl acetate were added to vial **D**.
7. 2.5ml ethyl acetate were added to vials **E-J**.
8. 0.8ml **C** were transferred to **D**, and mixed well, = 800pg 100μl<sup>-1</sup> (**D**)
9. 2.5ml **D** were transferred to **E**, and mixed well, = 400pg 100μl<sup>-1</sup> (**E**)
10. 2.5ml **E** were transferred to **F**, and mixed well, = 200pg 100μl<sup>-1</sup> (**F**)

11. 2.5ml **F** were transferred to **G**, and mixed well, = 100pg 100 $\mu$ l<sup>-1</sup> (**G**)
12. 2.5ml **G** were transferred to **H**, and mixed well, = 50pg 100 $\mu$ l<sup>-1</sup> (**H**)
13. 2.5ml **H** were transferred to **I**, and mixed well, = 25pg 100 $\mu$ l<sup>-1</sup> (**I**)
14. 2.5ml **I** were transferred to **J**, and mixed well, = 12.5pg 100 $\mu$ l<sup>-1</sup> (**J**)
15. The above working standard solutions were stored at -20°C.

#### *Assay buffer*

1. 200mg Bovine Serum Albumin (RIA grade, Sigma, D-4751) were weighed out into a 200ml Schott Duran<sup>®</sup> bottle. One Phosphate Buffered Saline (PBS) tablet (Sigma, P-4417) was added with 200ml distilled water.
2. The above was stirred using a magnetic stirrer.
3. The assay buffer was stored at 4°C.

#### *Dextran-Coated Charcoal (DCC)*

1. 200mg dextran (clinical grade, Sigma, D-4751) and 1g activated, neutralized charcoal (Sigma, C-5385) were added to a 200ml Schott Duran<sup>®</sup> bottle. One PBS tablet and 200ml distilled water were added.
2. The contents were mixed thoroughly using a magnetic stirrer.
3. The resulting DCC suspension was stored at 4°C.

#### *Antibody*

1. Anti-cortisol (rabbit polyclonal, Abcam plc) was used at 1:1500 final dilution.

## **Assay Procedure**

### *Extraction of plasma samples*

1. Steroids were removed from aqueous samples by extraction with ethyl acetate.
2. 200µl plasma were transferred by pipette into labelled, capped 1.5ml centrifuge tubes. *[Variations of sample volume were possible, providing the ratio of solvent to sample was maintained at 5:1].*
3. 1.0ml ethyl acetate was added to each sample.
4. Each sample tube was capped and vortex mixed thoroughly.
5. Each sample was centrifuged for 2 minutes to separate the aqueous and solvent phases.

### *Construction of standard curve*

1. 16 3.5ml PP (polypropylene) assay tubes were labelled 1-16.
2. To tubes 1 and 2 were added 100µl ethyl acetate only.
3. To tubes 3 and 4 were added 100 µl of standard inert cortisol solution **J**
4. To tubes 5 and 6 were added 100 µl of standard inert cortisol solution **I**
5. The above was repeated for the remaining standard solutions **H-D** and assay tubes 7-16.

6. To all tubes 1-16, 50 µl of 3H-cortisol working solution were added, using a repeater pipette

*Transfer of unknowns to assay tubes*

1. An appropriate number of 3.5ml PP assay tubes were labelled from 17 onwards, according to number of unknown samples.
2. 150µl of sample was transferred via pipette into the appropriate assay tube.
3. 50 µl of 3H-cortisol working solution were added to all sample tubes, using a repeater pipette.

*Evaporation of ethyl acetate*

1. The rack(s) containing the assay tubes were transferred to the vacuum oven (Gallenkamp), heated to 40°C.
2. The oven door was closed, inlet valve opened and the vacuum pump attached.
3. The pump was switched on, and the pump valve closed when the extracts had dried.
4. The pump tubing was removed, pump switched off, and inlet valve slowly opened.

*Addition of buffer and antibody*

1. To each tube was added 100 µl of assay buffer, using a repeater pipette.
2. To each tube 100 µl antibody solution was added, using a repeater pipette.

3. The solutions were mixed well, the racks covered with cling film, and the tubes incubated overnight (minimum 6 hours, maximum 18 hours) at 4°C.

#### *Addition of DCC*

1. The centrifuge (Hettich Rotanta 460R) was switched on 30 minutes prior to this stage of the assay to allow the chamber to cool to 4°C.
2. Scintillation vials were pre-filled with scintillation fluid (4 ml), arranged in racks, and caps should be labelled and arranged on bench ready for use.
3. When incubation period was complete, the racks of tubes were placed on ice.
4. The cling film was removed, and 100 µl of stirred, chilled DCC added to each tube using a repeater pipette. *[The tip of the pipette nozzle was cut off to prevent clogging by charcoal granules.]*
5. Each tube was vortex mixed and incubated on ice for 5 minutes.
6. The tubes were transferred to the centrifuge buckets. The lid was closed and the centrifuge started.
7. The DCC was spun down (3,500 rpm for 5 minutes, centrifuge cooled to 4°C).
8. The tubes were transferred from the centrifuge buckets to the racks on ice.
9. 200µl of the supernatant was carefully transferred by pipette from each tube to a scintillation vial, and each tube loosely capped.
10. When all samples had been transferred, the caps were firmly pushed into place on the tubes, and the contents mixed by repeated inversion.



11. The vials were transferred to the scintillation counter and the samples counted under standard tritium conditions for >5 mins.

*Assay calculation*

1. The completed data set was transferred from the counter to a PC.
2. SigmaPlot Regression Wizard (Systat Software Inc) was used to fit a curve (3-parameter hyperbolic decay) to the standards (x axis = pg steroid/tube; y axis = % <sup>3</sup>H steroid bound).
3. The equation from this curve was used to derive the concentration of steroid in the unknown samples:  $x = [(a*b)/(y-c)]-b$
4. Corrections were made as appropriate for volume of sample assayed to provide a final estimate as ng steroid / ml plasma.