THE EFFECTS OF MATERNAL STEROIDS ON INDIVIDUAL VARIATION IN JUVENILE SALMONIDS

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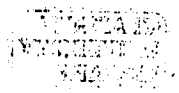
ABSTRACT

- Understanding the basis of individual variation is a central theme in biology, as selection acts upon such variation. The genetic contribution to variation is well established, as are environmental influences upon the expression of individual genotype.

- A special (and particularly complex) form of environmental effect that is often overlooked, is the transfer of non-genetic materials to offspring via the yolk or placenta. The amount and nature of materials transferred is influenced by maternal phenotype and environment. Recently, attention has turned to the effects of maternal steroids on offspring physiology and behaviour in a range of taxa.

- The research described in this thesis examined whether concentrations of maternal steroids in the egg at fertilisation can influence ecologically important physiological and behavioural traits in juvenile salmonids. I have addressed four questions: Can experimental manipulation of egg steroid concentrations influence offspring physiology and behaviour? What is the extent of natural variation in the steroid content of a female's eggs at fertilisation? Does natural variation in egg steroid content influence offspring phenotype? Does maternal social status influence maternal and egg steroid concentrations?

- Experimental elevations of the cortisol and testosterone content of brown trout eggs (Chapter 2) indicated that concentrations of these hormones may influence juvenile size, resting metabolic rate and social status. However, there was great inter-family variation in the effects of treatment, and the possibility that the variation observed is due to differences in rearing environments rather than treatment is an equally plausible hypothesis. Thus, maternal steroids in the egg at fertilisation may be able to influence aspects of juvenile physiology and behaviour that are associated with early competitive ability and survival, but so too may undetected variation in the rearing environment.



- To determine the scope for an effect of maternal steroids in the eggs, I then investigated the degree of intra-female variation in egg steroid content, both before (Chapter 3) and after ovulation (Chapters 3 & 4). Before ovulation, follicle cortisol content and weight varied between different regions of the ovary, but patterns of variation were not consistent between females. In some cases when females were allowed to spawn naturally, egg steroid content varied between nests deposited by the same female, but patterns of inter-nest variation were not consistent between the eggs of different females. I suggest that the steroid content of ovulated eggs can change while eggs are retained in the body cavity, resulting in inter-nest variation. The magnitude of inter-nest variation in egg cortisol concentrations was equivalent to that achieved in the experimental manipulation study, suggesting that natural variation in egg cortisol concentrations might influence offspring phenotype if the effects observed in Chapter 2 were due to treatment and not 'tank effects'.

- Effects of natural variation in egg steroid content, and of spawning order, were investigated in offspring reared from each nest spawned by seven pairs of wild brown trout. There were weak indications that eggs spawned into the first nest tended to be more viable during embryogenesis than eggs spawned into later nests (Chapter 4). In three of seven families, earlier nests were also found to hatch significantly earlier than later nests (Chapter 6). In some cases, inter-nest variations in the stress physiology of alevins were found, but it seems unlikely that this variation is related to egg steroid content (Chapter 5). As in Chapter 2, it is possible that the variation observed was a consequence of undetected differences in the rearing environment. Offspring from different nests could also vary in their juvenile size, but tank effects and high mortality prevented close examination of variation in juvenile physiology and behaviour (Chapter 6).

- The effects of female social status on ovarian development were examined to see whether social interaction could influence egg growth, and plasma and egg steroid levels (Chapter 7). This experiment was confounded by disease and loss of identification tags, but sufficient information was gathered to determine that maturing females form aggression-based social hierarchies that were stable over at

least short periods of time. There was a negative association between plasma levels of the stress hormone cortisol and concentrations of vitellogenin. Further study of the effects of social status on broodstock is required.

- The high degree of inter-family variation and sensitivity to rearing environment described here has implications for experimental design and data interpretation.

- While I have gathered sufficient information to suggest a possible role of maternal steroids in the egg on offspring physiology and behaviour, further research at both molecular and population levels is required to test this and to determine the ecological relevance of this very complex system.

CANDIDATE'S DECLARATION

I declare that the work recorded in this thesis is entirely my own, and of my own composition. No part of this work has been submitted for any other degree.

Hayley Suter
November 2002

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General Introduction

Individual variation and maternal effects

Understanding the basis of individual variation is a central theme in biology, as selection acts upon such variation. The genetic contribution to variation is well established, as are environmental influences upon the expression of individual genotype. A special (and particularly complex) form of environmental effects that are often overlooked, are *maternal effects*.

Maternal effects occur when the phenotype of an individual is determined not only by its own genotype and the environmental conditions it experiences, but also by the phenotype or environment of its mother (Kempthorne 1969, Wright 1969, Falconer 1989, Wade 1998). Because the mother's gamete is larger than that of the father, mothers tend to have a greater environmental impact on offspring development. The maternal phenotype, especially maternal nutritional condition, can affect gamete size, 'quality', and the materials taken up by offspring during development (Wade 1998). If developmental environment (i.e., nest site or maternal habitat during gestation) is also under maternal control and affects offspring phenotype, then this is yet another form of maternal effect. Thus, in many instances, the ecology of the maternal environment and maternal phenotype can have important effects on offspring development and subsequent fitness. Maternal effects have been reported in insects, fish, amphibians, reptiles, birds and mammals, and several excellent reviews have been written on the subject (see Mousseau & Fox 1998).

Maternal effects on egg size have received the most attention of the potential non-genetic maternal contributions to offspring (Bernardo 1996). In many animal groups, offspring are entirely dependent on the contents of the egg for fuel and building materials during development. Thus small eggs contain less energy for developing offspring. However, mothers transfer not only large amounts of lipid to offspring via the egg or placenta, but proteins, enzymes, RNA, antibodies, hormones, and a range of other bioactive compounds also (Mommsen & Walsh 1988, Reznick 1991, Kamler 1992, Brooks *et al.* 1997, Mousseau & Fox 1998, Blount *et al.* 2000, Schreck *et al.* 2001). Any of these materials could theoretically influence offspring phenotype.

Effects of maternal hormones on offspring

The effects of exposure to hormones during development have been well documented in several animal taxa (Mousseau & Fox 1998). Exposure to androgens and stress-related hormones while *in utero* has been demonstrated to influence the behaviour and physiology of several mammals (see review by Clark & Galef 1998). For example, experience of social stress during pregnancy in guinea pigs (*Cavia aperea* f. *porcellus*) results in adult female offspring that display behaviours characteristic of males (Sachser & Kaiser 1996) and adult male offspring with a diminished physiological stress response and protracted period of infantile behaviour (Kaiser & Sachser 2001). Among egg layers, the amount of maternal testosterone in bird eggs can vary within a clutch, and have significant effects on offspring fitness. Schwabl (1993) demonstrated that the amount of maternal testosterone in canary (*Serinus canaria*) eggs increased with laying order. Increased yolk testosterone was associated with higher begging frequencies and growth rates after hatch, presumably reducing the disadvantages associated with late hatching within a clutch (Schwabl 1996). A similar relationship between laying order and egg testosterone content was found in the red-winged blackbird (*Agelaius phoeniceus*), with higher testosterone levels being found to enhance the development of the hatching muscle in this species (Lipar & Ketterson 2000).

Maternal hormones and fish

Large amounts of maternal steroid and thyroid hormones are also found in teleost eggs (Kobuke *et al.* 1987; Brown *et al.* 1988, Feist *et al.* 1990, de Jesus & Hirano 1992; Hwang *et al.* 1992). Experiments have demonstrated that changes in maternal plasma hormone concentrations (through administration of exogenous hormones or in response to manipulations of the maternal environment) can affect the concentration of those hormones in the egg. For example, Brown *et al.* (1988) injected female striped bass (*Morone saxatilis*) with triiodothyronine (a thyroid hormone), and observed an increase in the concentration of this hormone in the egg. Stratholt *et al.* (1997) stressed female coho salmon (*Oncorhynchus kisutch*) by chasing them within the tank with a hand net during the final stages of ovarian development. This resulted in an elevation in maternal plasma cortisol concentration, the typical endocrine response to a stressor, and stressed females produced eggs with significantly higher cortisol levels than control fish. Thus factors affecting maternal endocrine status can affect the

hormone content of eggs. But what evidence is there for an effect of maternal hormones on the development, physiology and behaviour of their offspring?

In all fish species examined, levels of hormones in the egg decrease prior to the onset of endogenous hormone production by the embryo (Kobuke *et al.* 1987, Rothbard *et al.* 1987, Tagawa & Hirano 1987, 1990, Leatherland *et al.* 1989, Feist *et al.* 1990, de Jesus & Hirano 1992, Leatherland & Barrett 1993, Feist & Schreck 1996). Using radioactive precursors, it has been shown that salmonid embryos are capable of metabolising maternal steroids in the yolk very early in development (Pillai *et* al. 1974, Antila 1984, Yeoh *et al.* 1996b, Khan *et al.* 1997a,b,c). Thus the developing fish is probably responsible for the decline in egg hormone concentrations prior to the onset of endogenous hormone production. But do maternal hormones have a role in early development, or are they simply being absorbed and excreted by the embryo as a by-product of yolk absorption? If concentrations of maternal hormones affect larval survival or growth, then this system could be manipulated to improve the productivity of aquaculture systems. This possibility provided the impetus for studies examining the effects of early exposure to hormones on the early development of offspring.

Thyroid hormones

Several studies have focused on the actions of thyroid hormones (triiodothyronine and thyroxine) because of their role in development in a range of vertebrates (McNabb & King 1992). In the manipulation experiment described above, Brown *et al.* (1988) found that triiodothyronine treatment of female striped bass (and hence elevated triiodothyronine levels in eggs) resulted in a higher rate of swimbladder inflation and increased survival. Thyroxine injections of female rabbitfish (*Siganus guttatus*) broodstock had similar effects, elevating maternal hormone levels and increasing larval growth and survival (Ayson & Lam 1993). Dales & Hoar (1954) manipulated the amount of thyroxine in the eggs of chum salmon (*Oncorhynchus keta*). Addition of thyroxine accelerated growth of the body wall and pectoral fins during early development, but reduced the rate of increase in body length. Depletion of egg thyroxine levels also reduced the rate of length increase, but had no other effects. In contrast, Tagawa and Hirano (1991) found that experimentally depleting thyroid hormone levels in Japanese medaka (*Oryzias latipes*) eggs by more than 90 % (by reducing thyroid hormone levels in mothers prior to spawning) had no effects on larval

survival or growth. They concluded that the thyroid hormones removed from the eggs were not essential for early development in medaka.

These studies suggest that there may be species differences in mechanisms of storage and utilisation of maternal hormones, and possibly in the levels of thyroid hormones required by embryos during early development. In the medaka, 10 % of the thyroid hormones contained in an egg may be sufficient to meet the needs of the embryo, while in the striped sea bass the concentration of thyroid hormones in the egg may be the factor limiting some aspects of development. Looking at thyroxine levels in coho salmon embryos, Kobuke *et al.* (1987) found that levels remained fairly constant to hatching, at which time the yolk contained 96 % and the embryo 4 % of thyroxine present. While it is possible that natural levels of thyroxine (as opposed to the effects of artificially elevated levels; Warner 1952, Dales & Hoar 1954) do not play a significant role in embryogenesis, it is also possible that this 4 % redistribution from yolk to embryo may be sufficient for embryonic development (Specker 1988).

Sex steroids

Another area of great interest to larvaculture is the manipulation of sex ratios. In salmonids, female stocks are desirable as juvenile males may mature rather than concentrate on somatic growth (Zohar 1989). In tilapia (*Oreochromis mossambicus*), male stocks are desirable as males grow faster (Zohar 1989). Administering sex steroids during early development can easily reverse both sexes (reviewed by Yamamoto 1969; Schreck 1974; Donaldson & Hunter 1982; Hunter & Donaldson 1983; Francis 1992). Levels of androgens and progestogens may be quite high in newly fertilised salmonid eggs (Feist *et al.* 1990; de Jesus & Hirano 1992), but levels decline towards hatching. Sexual differentiation in salmonids is believed to commence around this time (Feist & Schreck 1996). Feist and Schreck (1996) suggest that if sex steroids are influential in sex differentiation in rainbow trout (*O. mykiss*), then those steroids are therefore more likely to be of embryonic origin rather than maternal. This is not to say that sex steroids within the yolk do not affect other developmental processes, or that other compounds within the yolk do not affect sex differentiation. Carlson *et al.* (2000) investigated gonadal development in rainbow trout embryos microinjected with organochlorines. Sexual development was not consistently altered by exposure to endocrine-active chemicals, but occasional anomalies were found.

Conceivably, maternal exposure to pollutants could alter egg composition and affect offspring development.

Cortisol

Could maternal hormones in the yolk, like some pollutants, have detrimental effects upon larval development or offspring fitness? Not surprisingly, studies that have looked at this area have concentrated on the effects of cortisol, a corticosteroid hormone elevated in the plasma when fish are stressed (Barton & Iwama 1991). The effects of experimentally elevated levels of maternal plasma cortisol in relation to ovarian development, fecundity, egg size, egg viability, larval metamorphosis and susceptibility of juveniles to disease have been examined (Campbell *et al.* 1992 & 1994; Foo & Lam 1993; Stratholt *et al.* 1997; Contreras-Sanchez *et al.* 1998; McCormick 1998; Lethimonier *et al.* 2000 – see reviews by Leatherland 1999 and Schreck *et al.* 2001). In these experiments, maternal plasma cortisol concentrations were elevated either directly through implantation, or indirectly through exposure to some sort of physical stressor such as netting or confinement. Other studies have examined the effects of post-fertilisation exposure to cortisol on larval growth, metamorphosis and survival (Brooks *et al.* 1995, Brown & Kim 1995; Stratholt *et al.* 1997, Lin *et al.* 1999; McCormick 1999 – again, see reviews by Leatherland 1999 and Schreck *et al.* 2001).

Results from maternal manipulation studies have proved inconclusive, probably due to the different stressors used, and confounding environmental and husbandry factors (Foo & Lam 1993; Leatherland 1999). Studies manipulating post-fertilisation yolk cortisol levels by immersion or microinjection avoid the 'noise' associated with broodstock experiments, and have demonstrated that exogenous cortisol can affect larval development. For example, Brown and Kim (1995) reported that post-hatching immersion in thyroid hormones and cortisol significantly accelerated the onset of initial gut formation and somatic pigmentation in the Pacific threadfin (*Polydactylus sexfilis*) over rates seen in control fish and fish treated with cortisol or thyroid hormones alone. In contrast to this positive role for cortisol, McCormick (1999) found that Ambon damselfish (*Pomacentrus amboinensis*) eggs immersed in cortisol from fertilisation to hatching (4.5 days) showed reduced larval length at hatching. Levels of

cortisol within the eggs during treatment were within the physiological range for eggs of that species (McCormick 1999).

Leatherland (1999) reviewed the effects of stress and cortisol on reproduction in salmonids. He drew attention to the fact that plasma cortisol is often elevated in female salmonids during late sexual maturation, and that cortisol levels would be expected to be high in the eggs of those species. He suggested that if the gonads are sensitive to cortisol levels during late development, then impaired egg quality would be expected in these salmonid species. There is no evidence for this. Further, although egg cortisol content of salmonids can be influenced by changes in plasma cortisol levels in the female, there is no correlation between egg cortisol content and embryonic development or mortality.

Brooks *et al.* (1995) and Stratholt *et al.* (1997) elevated egg cortisol concentrations by immersing newly-fertilised eggs in water baths containing cortisol. They found no significant effect of elevated egg cortisol levels on rainbow trout or coho salmon development or survival, and found that cortisol cleared rapidly from eggs, leading them to suggest that maternal cortisol is cleared from the egg before hatching and may not be a factor in later development. However, neither of these studies considered possible post-larval consequences of treatment; cortisol absorbed by the embryo during early development could influence the behaviour and physiology of juvenile or adult salmonids. In addition, given that the egg is essentially a closed system until hatching, with limited permeability, cortisol metabolites or conjugates are likely to be retained within the egg in high concentrations. These compounds could have some effect on development. In experiments where egg cortisol content is manipulated through exposure of the female to an environmental stressor, it is also possible that the type of stressor may affect levels of other chemicals deposited in the egg, and these chemicals could have an effect on development.

In summary, very little is currently known about the effects of maternal egg hormones on early development in fish, but there is reasonable evidence to suggest that they could play a role. In marine species, it appears that cortisol and thyroid hormone concentrations can affect larval development and survival. In salmonids, levels of thyroid hormones in the egg may affect early development, but no effects of sex

steroid or cortisol concentrations in the egg at fertilisation have been observed. However, these studies have often involved pharmacological elevations of hormone levels, have considered very few aspects of offspring physiology, and have not considered offspring behaviour at all. It is entirely possible that natural variation in egg hormone concentrations could influence the behaviour and physiology of juvenile fish, and contribute to individual variation in ecologically important traits. If this is the case, then environmental factors affecting maternal hormone status could influence offspring characteristics through effects on egg hormone concentrations.

Steroid-mediated effects of maternal environment on offspring: a case study

One of the most recent studies in the 'maternal effects' field investigated the mating behaviour of small tropical damselfish on a natural reef. McCormick (1998) demonstrated that cortisol levels in female damselfish during breeding were related to levels of intrasexual competition and the predation risk. Thus individual females may vary markedly in their hormone levels depending on their environment. In turn, McCormick (1998) found that the size of larvae at hatching significantly varied between mothers, and that levels of maternal cortisol could explain much of the variation in progeny size. By manipulating levels of maternal cortisol through implantation, he was able to show an inverse relationship between cortisol levels (maternal and egg) and larval length. He has further demonstrated that post-fertilisation exposure to testosterone and cortisol can significantly affect larval growth (McCormick 1999). It was suggested that, by affecting the course of larval development, the concentration of hormones in the eggs could influence larval mortality, and thus which individuals survived to produce the next generation (McCormick 1999). Is a similar mechanism present in other fish groups? This thesis investigates whether maternal experiences and competition for resources could, via the transfer of steroids to the egg prior to fertilisation, generate phenotypic variation in juvenile salmonids.

The study system: salmonids

Individual salmonids vary greatly in their physiology, behaviour, and life history strategies (see Marschall *et al.* 1998, Metcalfe 1998, Thorpe *et al.* 1998, Nislow 2001, Sloman & Armstrong 2002). Understanding the causes and patterns of individual variation is important in the study of salmonids in natural habitats, and is also central

to improving fish welfare and productivity in aquaculture systems (Turnbull *et al.* 1998, MacLean *et al.* 2000). Many of the causes of individual variation in juvenile salmonid physiology and behaviour are not clear, but maternal effects may play a role.

As background to the salmonids and the question of whether transfer of maternal steroids to offspring has developmental consequences, I briefly review female salmonid reproductive biology and behaviour, and the ecology of juvenile salmonids, before introducing the contents of this thesis. The word 'salmonids' is used as a generic term throughout this thesis, but the research described in this thesis is predominantly conducted on brown trout, *Salmo trutta* L.

The reproductive biology of female salmonids

Oocyte growth and maturation

The recruitment, growth and maturation of oocytes have been extensively and excellently reviewed by several authors (e.g., Wallace & Selman 1981, Nagahama 1987, Mommsen & Walsh 1988, Billard 1992, Nagahama *et al.* 1993, Tyler & Sumpter 1996, Wiegand 1996). A very brief and simplified outline of the process of oocyte growth and maturation is as follows. The hypothalamus is stimulated to produce gonadotropin-releasing hormone (GnRH). GnRH is transported to the pituitary gland where its primary function is to stimulate the production and release of gonadotropins (GTHs). GTHs enter the blood system and circulate throughout the body, the major target organ being the gonads. During the main period of oocyte growth (known as *vitellogenesis*), GTH binds to the cellular sheath that surrounds each oocyte during development (known as the *follicle layer*) and stimulates the production of the sex steroid *testosterone*, and its conversion to *estradiol*. Estradiol is carried through the blood system to the liver, where it stimulates the production of yolk (aka: *vitellogenin*). Several endocrine feedback circuits regulate this process. Vitellogenin, a large glycophospholipoprotein, moves from the liver to the ovaries via the bloodstream and is taken up into the oocytes by micropinocytosis.

Towards the end of vitellogenesis, there is a switch in the kind of GTH produced and released from the pituitary gland, and a corresponding steroidogenic switch within the follicle layer of the oocytes. The follicle layer begins to produce a maturation-

inducing steroid that binds to the oocyte membrane and triggers the resumption of meiosis – a process known as *final oocyte maturation.* In the salmonids, this maturation-inducing steroid is 17α,20β-dihydroxy-4-pregnen-3-one, a progesterone derivative. Final oocyte maturation involves the expulsion of a set of chromosomes to allow the gamete to become haploid. Around the time of final oocyte maturation, the ovaries begin to break down as oocytes are ovulated (released from the follicle layer) into the abdominal cavity. The eggs may be fertilised once final oocyte maturation has taken place. In salmonids, the process of oocyte development through to spawning takes several months.

Breeding behaviour

Several authors (Jones 1959; Tautz & Groot 1975, Webb & Hawkins 1989; Fleming 1996, 1998) have described the breeding behaviour of female salmonids. Reproductively mature females move into areas of fast moving water and gravel substrata, and choose an area in which to commence nest-building activities. They use their caudal fins to dig nests in gravel areas, creating hollows into which they release eggs for fertilisation. Following oviposition, the female immediately starts covering the nest with gravel from the nest margins, often creating the next nest in which she will spawn. This process may be repeated multiple times, and the area of gravel within which nests are constructed is referred to as a *redd.* Successive oviposition events are usually less than a day apart, although the time between such events may range from four hours to nine days in Atlantic salmon *Salmo salar* (Fleming 1996). The number of eggs a female deposits in each nest varies directly with her size and inversely with the number of previous ovipositions she has made (Fleming 1996). Female salmonids usually complete spawning within five or six days, during which time they may aggressively defend their nesting territories. Ongoing defence of the redd after the completion of spawning is observed in some *Oncorhynchus* species, but is absent from the behavioural repertoire of other salmonids such as the Atlantic salmon (Fleming 1996, 1998).

The females decision of where to spawn appears to be affected by several factors, including habitat conditions, local adaptation, traditionality and intrasexual competition (Fleming 1998). Active choice of breeding locality may be constrained by

overt aggression from other nesting females (Jones 1959; Webb & Hawkins 1989; Fleming *et al.* 1997). Intraspecific competition may also affect the timing of reproduction. Early spawning females may have ready access to the highest-quality breeding spawning sites, but these nests may be disturbed by the digging activity of later-spawning females.

Female body size can influence access to, and defence of, preferred nesting sites in many salmonid species, and can affect nest quality (bigger fish can dig deeper nests). Larger salmonids also produce larger eggs than small females (reviewed in Fleming 1996). These larger eggs often give rise to larger juveniles (Glebe *et al.* 1979; Kazakov 1981; Thorpe *et al.* 1984, Hayashizaki *et al.* 1995) which may be competitively advantaged and have higher survival (e.g., Bagenal 1969; Wankowski & Thorpe 1979; reviewed in Fleming 1996). Advantages associated with larger egg size may be compromised by increased mortality during incubation resulting from poor intergravel oxygen conditions (van den Berghe and Gross 1989, Quinn *et al.* 1995).

Fertilisation and early development

As briefly alluded to above, fertilisation of eggs in salmonids takes place externally. In many fish species, the egg membrane hardens following the fertilisation of ova. In salmonids, however, the egg membrane rapidly hardens on contact with freshwater, so fertilisation must occur immediately after oviposition (Billard 1992). The zygote goes through meroblastic cleavage and the embryo slowly develops on top of the energy-supplying yolk sac (Heming & Buddington 1988). At some point in development, the egg hatches. This developmental stage varies between species, and can be affected by a wide range of environmental variables (reviewed by Kamler 1992). Hatching involves the emergence of the embryo following the dissolution or softening of the egg envelope by an embryo-secreted hatching enzyme (Yamagami 1988). Following hatching, the larvae live off the energy-rich contents of the yolk sac (Heming & Buddington 1988). Embryonic and larval development of salmonids takes place in the redd, with the fish emerging from the gravel around the time of complete yolk-sac absorption (Huntingford 1993).

The ecology of juvenile salmonids

Fish biologists refer to different salmonid life stages by specialised names, which I now define for the purposes of this thesis. While salmonid larvae are generally known as *alevins*, after emergence they are referred to as *fry* until absorption of the yolk-sac is complete. In migratory salmonid species, the post-fry freshwater juvenile is known as a *parr*, but I try to avoid using this term, as my use of the word 'salmonids' includes both migratory and non-migratory fish. Instead, I refer to fry and parr as *juveniles* throughout this thesis.

Feeding ecology and behaviour

Soon after salmonids emerge from the redd, they begin to display aggressive behaviours and compete for access to resources such as food and shelter (Kalleberg 1958, Mason & Chapman 1965). Juvenile salmonids are largely sit-and-wait predators, holding position at a feeding station and darting out to pluck invertebrates drifting past in the water column (Stradmeyer & Thorpe 1987). Fish will tend to aggressively exclude other fish from the area around their feeding station (generally termed *territory*), and are therefore able to monopolise invertebrate drift within that territory. However, foraging and territorial behaviour will vary with the density of juveniles and the carrying capacity of the environment (as defined by food and habitat availability) (Ruxton *et al.* 1999). If there is a surfeit of suitable feeding positions, all individuals are likely to have one or more feeding stations, and time spent in defence of feeding territories may be low. If the carrying capacity of a stream reach is exceeded, then individual ability to acquire resources relative to other fish will determine individual success. In a hatchery situation, resources are generally not limiting, but predictable food delivery regimes can lead to monopolisation of the resource by more competitive individuals (Thorpe *et al.* 1990, Alanärä & Brännäs 1996), having implications for both fish welfare and farm productivity (Jobling 1995, Turnbull *et al.* 1998, MacLean *et al.* 2000).

Individual variation in competitive ability

Individuals vary in their ability to compete for territories, shelter and food (Fausch 1984; Huntingford & Garcia de Leaniz 1997; Kadri *et al.* 1997, Cutts *et al.* 1999a), often leading to the development of hierarchical social structures in both wild and tank environments (e.g., Keenleyside & Yamamoto 1962; Wankowski & Thorpe 1979;

Adams *et al.* 1998). Competitive ability is generally positively associated with aggressiveness, and may be of particular importance immediately after emergence from the redd. This time presents a special set of challenges for juvenile salmonids. Fish must not only adjust to a complete change in their physical environment, but must also learn to feed and to evade a range of predators. Unsurprisingly, the period just after emergence is characterised by high juvenile mortality, as many fish starve (Elliott 1994, Garcia de Leaniz *et al.* 2000), are killed by predators (Brännäs 1995, Garcia de Leaniz *et al.* 2000), or encounter adverse environmental conditions (e.g., spate conditions or low temperatures). A fish is less likely to starve if it is able to acquire a profitable feeding territory but, at the point of emergence, fish will generally find themselves at high-densities. If territories are limited, the ability to acquire and defend a feeding station is therefore likely to have serious consequences for the future survival and growth of an individual. This may be particularly true in relatively unchanging physical environments, as aggression-based dominance hierarchies in such environments appear to be stable over time (Bachmann 1984, Abbott *et al.* 1985, Stradmeyer & Thorpe 1987). Variation in the ability to acquire and defend a territory may also influence the timing and occurrence of events such as migration. For example, offspring of a non-migratory female brown trout may adopt a migratory or non-migratory life history dependent on environmental conditions during early life (Jonsson 1985). In a river system in Norway, Jonsson (1985) found that brown trout juveniles that grew rapidly in freshwater were less likely to migrate to sea than slower growing siblings.

Factors influencing competitive ability

What factors generate individual variation in competitive ability at emergence? It has been suggested that aggressiveness has a genetic component, and that at least part of this genetic component may relate to metabolic processes (see reviews by Metcalfe 1998, Sloman & Armstrong 2002). Several laboratory-based experiments have now illustrated that fish with a higher metabolic rate appear to be more aggressive (Metcalfe *et al.* 1995, Cutts *et al.* 1999a, Yamamoto *et al.* 1998, McCarthy 2001), although it is possible that these fish are not more aggressive because of their higher metabolic rate, but because they are hungrier and are fighting for access rights (Metcalfe *et al.* 1995, Sloman & Armstrong 2002). Within groups of siblings, a high metabolic rate also appears to be associated with an earlier onset of exogenous feeding

(see Metcalfe 1998). In the wild, this would equate to an earlier time of emergence from the redd, which may confer certain benefits.

Although early emergence perhaps increases the risk of predation (Brännäs 1995) and exposure to unfavourable environmental conditions, early emerging fish may gain access to unoccupied territories and, once established, have a competitive advantage over the newcomer (e.g., Cutts *et al.* 1999a,b). If feeding conditions are favourable, early emerging fish may also gain a size advantage by the time later fish emerge, amplifying any competitive asymmetry due to metabolic differences. For example, Cutts *et al.* (1999a) demonstrated that larger fish with prior ownership of a territory won 92 % of territorial contests with a smaller intruder. Female salmonids will often spawn multiple nests within a single redd over a period of days. Emergence time is likely to vary between these nests as a result of different spawning times. Benefits of early emergence might be particularly great, therefore, for offspring deposited into the first nest. Early emergence (also referred to as *first feeding*) within a nest or batch of eggs has also been linked to increased genetic heterozygosity (Allendorf *et al.* 1983, McCarthy *et al.* 1996).

Laboratory experiments have demonstrated that greater body size also confers a competitive advantage to older juveniles during territorial fights, although this advantage can be modified by factors such as prior social experience and experience of the area contested (see review by Sloman & Armstrong 2002). However, when such experiments also considered metabolic rate, and controlled for body size, the acquisition of dominance over a territory correlated with resting metabolic rate but not body mass (Metcalfe *et al.* 1995, Yamamoto *et al.* 1998, McCarthy 2001). It is therefore likely that, as proposed by Huntingford *et al.* (1990), increased body size is a consequence rather than a cause of dominance. Levels of brain monoamines (Winberg & Nilsson 1992), growth hormone (Johnsson & Bjornsson 1994) and plasma cortisol (Sloman *et al.* 2001a) have also been linked to aggressive ability in juvenile salmonids, but individual variation in the concentrations of these hormones at emergence have not been considered.

A role for maternal effects?

While variation in metabolic rate in developing fish and juveniles may simply indicate variation in metabolism-related alleles (Metcalfe 1998), as phenotypes reflect both genotype and environment, slight variation in the composition or microhabitat of the egg could affect metabolic rate. In salmonids, these factors are largely under maternal control. As far as I am aware, relationships between maternal effects such as these and metabolic rate or competitive ability of juvenile salmonids have not been examined. Indeed, the entire question of how maternal effects influence aspects of salmonid biology other than egg size, survival to first feeding, and offspring size (see review by Heath & Blouw 1998) have been generally neglected. This is despite the recognised importance of maternal effects in other animal groups.

This thesis examines the possibility that maternal environment influences juvenile brown trout physiology and behaviour via variation in concentrations of maternal steroids in the egg.

In **Chapter 2** I examine the effects of artificial elevation of egg cortisol or testosterone concentrations on ecologically important aspects of juvenile physiology and behaviour such as metabolic rate, aggressiveness and juvenile size.

For egg steroid content to influence variation between full-sibling juveniles, it has to vary both between and among the eggs of females. I therefore investigate the extent and distribution of variation in egg steroid content within the ovaries of females just prior to spawning (**Chapter 3**). Given the relationships between egg size and offspring described earlier, I also ask whether egg size varies within a female.

Plasma steroid concentrations of female salmonids change dramatically during the course of spawning (Liley *et al.* 1986b). If eggs are responsive to changes in plasma steroid concentrations right up to the point at which they are spawned, then it is possible that eggs spawned into different nests by a single female differ in their steroid content. This possibility is addressed in **Chapter 4** by allowing pairs of wild brown trout to spawn within an artificial stream.

I reared a proportion of the eggs from each naturally-spawned nest until two months after the onset of feeding. In **Chapter 4**, I discuss nest-based differences in egg viability, and consider nest-based variation in alevin stress physiology in **Chapter 5**. In **Chapter 6** I examine relationships between spawning order and egg weight, hatching date, juvenile growth, and juvenile metabolic rate.

The question of whether maternal social status influences plasma steroid concentrations and ovarian development is addressed in **Chapter 7**.

The results of these experimental chapters are synthesised in **Chapter 8**, and their implications for salmonid ecology, future research directions, and experimental design are discussed.

CHAPTER TWO

MATERNAL STEROIDS INFLUENCE THE BEHAVIOUR AND PHYSIOLOGY OF JUVENILE BROWN TROUT

Abstract

I investigated whether artificial elevation of steroid (cortisol or testosterone) content of eggs influences the physiology and behaviour of juvenile brown trout *(Salmo trutta)*. Steroid concentrations were artificially elevated in five families immediately post-fertilisation. The effects of hormone treatment on juvenile metabolic rate, size, dominance behaviours and sex ratios were assessed by comparison with control groups from the same families. Cortisol and testosterone treatment appeared to affect all variables except sex ratios. If these effects are a consequence of treatment (and not a reflection of undetected differences in rearing environment), then treatment could affect dominance relationships directly, but also indirectly through effects on juvenile size. Treatment effects varied between families, suggesting that effects may be related to steroid interaction with other genetic or non-genetic parental contributions to the offspring, but the possibility that effects could be due to undetected differences in the rearing environment cannot be ruled out. I conclude that naturally occurring variation in levels of maternal cortisol and testosterone in brown trout eggs could lead to significant variation in ecologically important aspects of offspring physiology and behaviour, but that further work is required to determine whether or not this is the case.

Introduction

Populations of juvenile salmonids are characterised by a large degree of individual variation in several behavioural and physiological traits that can influence their life history strategies (Metcalfe 1998). For example, individuals vary greatly in their resting metabolic rate at the onset of exogenous feeding, and metabolic rate is associated with early social dominance (Metcalfe *et al.* 1995; Cutts *et al.* 1999a). Social dominance is generally associated with increased access to food (Fausch 1984; Hutchings 1997), and food availability (Jonsson *et al.* 1996) and juvenile development rate (Thorpe *et al.* 1984) have been demonstrated to affect subsequent fecundity in Atlantic salmon. Thus individual variation in one physiological trait at the start of exogenous feeding can influence subsequent reproductive success.

Factors influencing the development of individual variation in metabolic rates, aggressiveness and foraging behaviours at first feeding are not well understood, but parental differences are likely to be involved. Parents contribute genetic and non-

genetic material to their offspring via the gametes. The phenotype of individuals at emergence will depend on the interaction between these parental contributions and the environment during early development (Mousseau & Fox 1998). Because genotype, physiology and environment vary between individual adults, the material they pass on in gametes will also vary (Brooks *et al.* 1997; Heath & Blouw 1998). In the case of teleost eggs, males generally contribute little more to zygotes than genetic material, while females contribute genetic material and a vast array of non-genetic material (Tyler & Sumpter 1996; Wiegand 1996; Brooks *et al.* 1997; Heath & Blouw 1998).

Several studies over the last 15 years have found large quantities of hormones in newly fertilised teleost eggs (Rothbard *et al.* 1987; Tagawa & Hirano 1987; Feist *et al.* 1990; Tagawa *et al.* 1990; Hwang *et al.* 1992; Stratholt *et al.* 1997). Concentrations of these hormones tend to vary between eggs of different females (Stratholt *et al.* 1997; Leatherland 1999). From experiments manipulating concentrations of thyroid and steroid hormones in female broodstock, it is known that hormones present in the eggs at spawning are of maternal origin (Brown *et al.* 1988; Ayson & Lam 1993; Brown & Kim 1995). The metabolism of yolk-sac steroids in developing embryos prior to the onset of endogenous hormone production has been demonstrated in several species (Pillai *et al.* 1974; Antila 1984; Yeoh *et al.* 1996a,b; Khan *et al.* 1997a,b,c), leading to the suggestion that maternal steroids in the yolk may play a role in the endocrine control of early development (Lam 1994; Brooks *et al.* 1997). If concentrations of these hormones are important in early development, then maternally-dependent variation in egg hormone concentrations could result in individual variation in metabolic rates, aggressiveness and foraging behaviours at first feeding.

In species in which the eggs are small and the period of embryonic and larval development is relatively short, manipulations to increase cortisol and thyroid hormone concentrations in the eggs have been shown to affect larval development (e.g., increased larval length and survival in the rabbitfish *Siganus guttatus* – Ayson & Lam 1993, increased survival and swim-bladder inflation rates in the striped bass *Morone saxatilis* – Brown *et al.* 1988). Recent studies of a tropical damselfish (*Pomacentrus amboinensis*) have demonstrated that maternal plasma and ovarian concentrations of cortisol are related to levels of predation risk experienced by the mother and to competition for mating opportunities (McCormick 1998), and that

elevated concentrations of cortisol have a negative impact on larval growth (McCormick 1999). These studies elegantly link population level variation in larval growth and development with the effects of environmental and social factors on the female, via the mechanism of varied egg cortisol concentrations. McCormick (1999) also investigated the effects of artificially elevated egg testosterone concentrations on damselfish larval development, and found that increased testosterone led to increased larval length.

Compared with most other teleosts, embryonic and larval development in the salmonids is long, and each developing fish is dependent on the contents of its yolk-sac as the sole source of energy for several months. While several studies have examined the effects of salmonid egg thyroid hormone concentrations at fertilisation on early development and survival (Dales & Hoar 1954; Kobuke *et al.* 1987; Tagawa & Hirano 1987; Mylonas *et al.* 1994; Raine & Leatherland 1999), the effects of steroid hormones such as cortisol and testosterone have received much less attention. Thyroid hormones are involved in the regulation of a number of processes within the female and probably enter the egg during vitellogenesis, the period of oocyte growth. Thyroid hormones have been shown to have a high binding affinity for vitellogenin in coho salmon *Oncorhynchus kisutch* (A Hara, WW Dickhoff & CV Sullivan, unpublished data; cited by Mommsen & Walsh 1988). Steroid hormones, on the other hand, are lipophilic and thus theoretically able to cross cell membranes and enter the egg at any stage prior to spawning. Among these, testosterone is an androgen primarily produced in females during vitellogenesis by cells in the follicle layer surrounding developing oocytes (Nagahama 1987). Cortisol, a corticosteroid, is commonly elevated during the physiological stress response in fish (Schreck 1981), and also during final stages of ovarian development in many salmonids (Leatherland 1999), including the brown trout, *Salmo trutta* (Pickering & Christie 1981).

The role of thyroid hormone levels in eggs during early development in salmonids is still unclear. Findings have been disparate, but do suggest that thyroid hormones have an influence on the developmental process (see Raine & Leatherland 1999). The few studies that have investigated the effects of egg cortisol concentrations have found no relationship between cortisol concentrations and survival or rate of development (Brooks *et al.* 1995; Stratholt *et al.* 1997; Leatherland 1999). The effects of salmonid

egg testosterone concentrations at fertilisation on aspects of development other than sexual differentiation have not been assessed. Studies on the effects of egg thyroid hormones and cortisol on juvenile salmonids have focused almost exclusively on growth, development and survival prior to first feeding. However, it is entirely possible that effects of maternal hormones in the egg may not become apparent until after the onset of exogenous feeding. Further, although research into the role of maternal thyroid hormones during early development is being advanced by a number of research groups, the potential roles of maternal steroids in salmonid eggs receive little attention.

This experiment examines whether physiological levels of steroids contribute to variation in physiological and behavioural parameters known to influence salmonid life history patterns, by artificially elevating cortisol or testosterone levels in brown trout eggs. By considering physiological and behavioural characteristics up to and beyond the point of first feeding, I extend the scope of previous studies. This experiment represents the first investigation of the effects of early embryonic testosterone levels on aspects of development other than sexual differentiation.

Methods

Broodstock, egg husbandry and hormone manipulations

Hormone manipulations were carried out at the Fisheries Research Services Freshwater Laboratory station at Almondbank, Perthshire, Scotland on 30 November 2000. Eggs were stripped from five mature female brown trout. The trout were 3-year old virgin spawners, second generation hatchery-reared from a non-migratory wild population. Forklengths and weights of females were measured before and after stripping. A gonadosomatic index (GSI) was calculated for each female by dividing the change in weight by the initial body weight. Blood samples taken from the caudal vein of each female after stripping were centrifuged, and the plasma transferred to clean containers and frozen for hormone analyses. A sample of ovarian fluid from each female was also frozen for analyses. Milt was collected from three mature males and pooled. Eggs were fertilised simultaneously with the pooled milt, then each of the five families was divided into three equal-sized groups and one group from each family treated with cortisol or testosterone, or used in a control treatment.

Control and experimental groups were placed in separate mesh baskets immediately after fertilisation and immersed for two hours in 5 l containers of water. The water, at ambient water temperature, contained 1 mg of cortisol (Sigma) or testosterone (Sigma) dissolved in 1 ml of 100 % ethanol, or 1 ml of ethanol alone (control). The final concentrations of ethanol and steroid were 0.02 % and 200 $\mu g.l^{-1}$, respectively. This method of manipulation has been shown to successfully elevate cortisol concentrations in salmonid eggs (Brooks *et al.* 1995, Stratholt *et al.* 1997).

During immersion, the treatment containers were regularly agitated to ensure that hormones in the water surrounding the eggs were not depleted. At the end of the two hours, the eggs were rinsed in fresh water and transferred to baskets in hatchery troughs flushed with running water (2 $l.min^{-1}$). To avoid cross contamination with steroids, separate troughs were used for eggs of each treatment for the first few days, but experimental and control groups were later arranged so that eggs from each female were in the same trough. This standardised the conditions experienced by control and experimental groups during development.

Eggs were sampled from each female before fertilisation, and from each group immediately after the 2-hour immersion period (n=5 samples.treatment^{-1}), and two and seven days (n=3 samples.treatment^{-1}) after fertilisation. Egg sampling involved blotting a sample of eggs dry on tissue, weighing them, and freezing pooled samples of around 200 mg in eppendorf tubes at –20 °C for later steroid analyses.

The change in individual egg weight as a consequence of hydration was not measured as part of this experiment. In the absence of such individual data, a % hydration value for eggs from each family was calculated as follows:

$$\% \text{ hydration} = 100(w_{2h} - w_0)/ w_0$$

where w_0 is the mean egg weight prior to fertilisation, and w_{2h} is the mean egg weight immediately after treatment (i.e., 2 h post-fertilisation).

Non-viable eggs (milky-opaque) were removed from each hatchery container 6 hours after treatment. The proportions of inviable eggs in each family, and in each treatment within families, were equivalent. Egg mortality rates between the day after treatment and hatching were very low in all groups (< 3%), and there was no mortality between hatching and first feeding.

Steroid extraction and analyses

Each egg sample was thawed at room temperature, and the egg membranes disrupted by hand using 20 strikes of a mounted needle. Sodium hydroxide (200 µl of 0.1M) was then added to each Eppendorf tube, and the samples vortex mixed for 30 s. After 1 h at room temperature, 1.0 ml of ethyl acetate was added to each Eppendorf tube. The tubes were capped and vortex mixed for 30 s, then centrifuged at 13000 rpm for 5 min to separate the phases. Most (0.9 ml) of the upper, solvent, phase was transferred into a clean, labelled Eppendorf tube, and a further 0.5 ml of ethyl acetate was added to the sample Eppendorf tube. After vortex mixing, the sample was again centrifuged, and 0.5 ml of solvent was removed into the same extract Eppendorf tube. To extract steroids from plasma and ovarian fluid samples, a known volume was transferred to a clean Eppendorf tube, and ethyl acetate was added and removed as described for the egg samples. Eppendorf tubes were capped between extractions to prevent solvent evaporation. Extracts were stored upright in sealed containers at –20 °C until steroid measurement. Extraction efficiencies for cortisol from eggs, plasma and ovarian fluid were (mean ± SE) 56.5 ± 0.4, 97.6 ± 0.3, and 95.7 ± 0.6 % (n=6). Extraction efficiencies for testosterone from eggs, plasma and ovarian fluid were (mean ± SE) 84.7 ± 0.5, 96.5 ± 0.4, and 98.6 ± 0.7 % (n=6). The accuracy of homogenisation and extraction procedures is demonstrated in Appendix 1.

Steroid concentrations in the extract were measured using radioimmunoassay, following the method of Pottinger & Carrick (2001) (Appendix 2). Serial dilutions of the extract showed that the dilution curve was parallel to the standard curve over the extract volumes used. Values were not corrected for extraction efficiency. Concentrations are expressed as $ng.egg^{-1}$ rather than $ng.g^{-1}$ egg as the concentration per egg shows the amount of steroid available to the developing embryo. This unit also

allows ready comparisons of steroid content before and after waterhardening, and between families.

Juvenile husbandry

Just prior to complete yolk-sac absorption, the 15 groups of fish (3 treatments x 5 families) were transferred to separate 1 m diameter hatchery tanks, and reared under identical conditions until testing (3-4 months beyond first feeding, when fish were 1-6 g). One family of fish was tested at a time in the following manner. Sixty fish from each treatment group were transferred to the Glasgow University Field Station at Rowardennan, Loch Lomondside. On arrival at the Field Station, the fish were anaesthetised, measured and a treatment-specific mark was tattooed on the fins using a needle (26 3/8 G) and Alcian blue dye. The treatment groups were then held in separate 0.5 m diameter hatchery tanks for four days prior to metabolic and behavioural testing. Fish were fed 4-5 times a day on commercial powder and crumb (Trouw). The U.K. Home Office Licence for this project (PPL 60/2025) required all fish to be killed by terminal anaesthesia (benzocaine, 100 $mg.l^{-1}$) and destruction of the brain following testing.

Metabolic rate measurements

Metabolic rate measurements involved placing individual fish into Perspex chambers through which oxygen-saturated water flowed at an adjustable rate. By measuring the depletion in oxygen between inflow and outflow under set conditions, the rate of oxygen consumption of each fish could be assessed (see Cutts *et al.* 1998 for details of set-up and calculations; Appendix 3). Oxygen consumption of resting fish is a direct estimate of resting metabolic rate (MR). Resting metabolic rate is exhibited by inactive fish in a postabsorptive state, and reflects costs of tissue maintenance and homeostasis. The MR of 20 individuals from each treatment group was measured. Oxygen consumption measurements were made at the same time each day to avoid confounding effects of circadian fluctuations in consumption rates. The fish were anaesthetised and measured at the end of the metabolic rate measurements.

To adjust for size-related differences in oxygen consumption, the residual resting metabolic rate (rMR) was calculated for each fish. The rMR is the difference between the observed value of oxygen consumption and the level of oxygen consumption

expected from the regression of oxygen consumption (ml $O_2.h^{-1}$) against weight (g) on a double natural logarithmic scale. Data from all families were combined in a single regression that yielded the following equation:

$\ln(O_2 \text{ consumption}) = 1.0385 \times \ln(\text{weight}) - 8.8033 \qquad r=0.652, p<0.001$

Behavioural observations

Eight enclosures were constructed against the viewing window of an artificial stream. Each enclosure was 30 cm long x 25 cm wide, and the water was maintained at a depth of 25 cm and a velocity of 1 bodylength.s^{-1}. The back walls of the enclosures were made of white Foamex, and white marble chips (approx. 0.5 cm diameter) were used to line the base. The ends of each enclosure were sealed with plastic mesh (3 mm mesh size) through which plankton moved freely.

A triad, comprising size-matched siblings, one from each treatment, was introduced into each section during the morning, and was observed at 2, 4, 8 and 24 h post-introduction. At each observation interval, the group was scanned (see below), then observed for ten minutes during which aggressive interactions and feeding were recorded, followed by a second scan. Following the observation at 24 hours, the fish were netted out into a bucket, terminally anaesthetised and weight and forklength measured. Twenty-four triads were tested from each family.

For each scan, each fish was given points for position (5 points if swimming, 2.5 points if resting, and 0 points if at the surface or tight against the enclosure sides) and body and eye colour were scored from 1 to 3 (3=pale, 1=very dark); several studies have indicated that darkened eye and body colour is characteristic of subordinate individuals (Keenleyside & Yamamoto 1962; Abbott *et al.* 1985; O'Connor *et al.* 1999, Suter & Huntingford 2002 – Appendix 4). During each 10-minute observation, each fish received 1 point for each aggressive interaction won, and 1 point if they were observed feeding on plankton.

A dominance score was calculated for each individual by totalling the position, colouration, aggression and feeding points for the four sampling intervals. Within each triad, the fish were ranked from 1 (dominant) to 3 (subordinate) by the magnitude of

the dominance scores calculated. The fish with the largest score in each triad was deemed to be dominant. In three triads, the scores of subordinate fish were tied, and the fish were assigned the rank 2.5.

Results from a pilot study during which eight triads were observed for five days suggested that observations made over a 24 h period following introduction to the enclosures was sufficient to identify the dominant individual. Specifically, in seven of the eight groups in the pilot study, a clear dominant was identified within 2 h of introduction to the enclosures, and these individuals maintained dominance for five days. In the eighth group, a clear dominant was not identified until 8 h after introduction to the enclosure. This individual maintained dominance to the end of the pilot study.

Sex ratios and juvenile size measurements

At the start of September 2001, the remaining fish (approximately 60 fish per treatment for each family) at Almondbank were killed, weight and forklength measured, and visually sexed following dissection. A condition factor (100 x weight/length^3) was calculated for each fish.

Results

Family variation in egg size and hydration

There were no significant differences between families in the mean wet weight of eggs at fertilisation (one-way ANOVA: $F_{4,152}$=2.12, p=0.081). Associations between maternal and egg characteristics were evaluated using correlations. A Pearson correlation coefficient of greater than 0.8783 indicated the presence of a significant association (two-tailed correlation with α=0.05 and n=5). These correlations must be regarded with caution given the small sample size. There was no obvious relationship between measures of female weight or forklength and egg weight at fertilisation, or between these measures of female size and degree of egg hydration during treatment. However, the female gonadosomatic index (I_G) was significantly positively related to post-treatment egg weight, and to the absolute amount of water absorbed during hardening (r=0.973 and 0.910, respectively; p<0.05). As there were no differences in

unfertilised egg weight between females, differences in I_G also reflect differences in relative fecundity (eggs.unit body weight^{-1}). So egg hydration may increase with increasing maternal relative fecundity.

Hormone concentrations

Hormone concentrations in eggs before and after treatment are shown in Table 2.1. Control egg steroid concentrations were not correlated with initial egg size, family differences in egg hydration, or with maternal plasma or ovarian fluid steroid concentrations at stripping. Changes in egg hormone concentrations from fertilisation to seven days post-fertilisation in each family are displayed in Figure 2.1. Control concentrations of cortisol did not change over time, and were not significantly different between families (two-way ANOVA with time and family as factors, $p>0.05$).

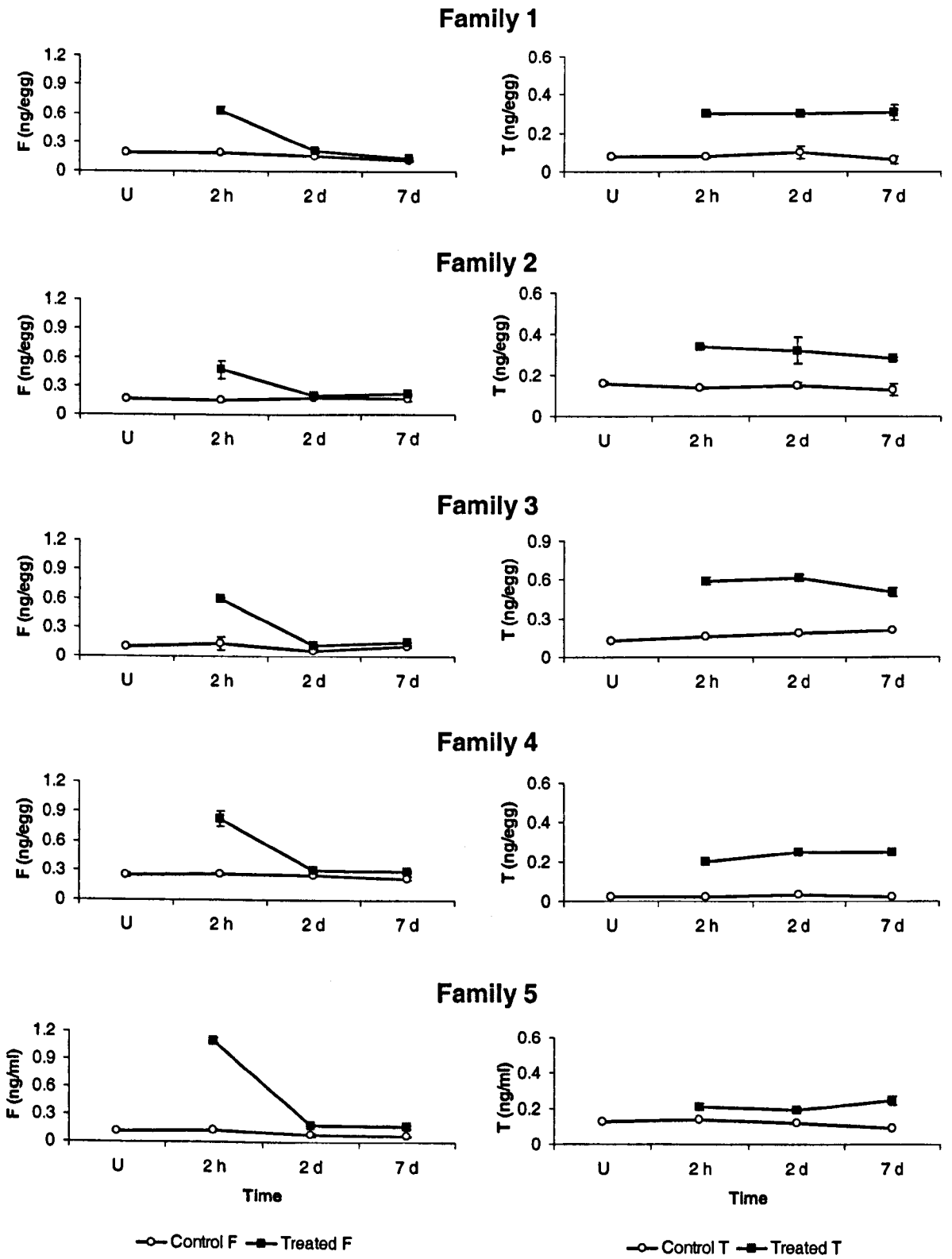
Control testosterone concentrations also remained constant up to seven days post-fertilisation, but varied significantly between families (Family effect: $F_{4,43}=79.28$, $p<0.001$). Control eggs from family 1 contained significantly less testosterone than eggs from families 2, 3, and 5, and significantly more testosterone than eggs from family 4 (Tukey's post-hoc comparisons of means).

Immersion of eggs in steroid-treated water for two hours immediately after fertilisation significantly elevated egg steroid concentrations above controls (2-sample *t*-tests, $p<0.05$). These elevations remained significant at seven days post-treatment. Testosterone concentrations remained constant over this time (two-way ANOVA of testosterone concentration by family and time: time, $F_{2,42}=0.16$, $p=0.854$), while cortisol concentrations dropped sharply from immediately post-treatment to two days post-treatment (two-way ANOVA: time, $F_{2,43}=252.23$, $p<0.001$). The amount of steroid uptake, and % increase in egg steroid concentrations, varied between families (Table 2.1.), but was not correlated with initial egg size or hormone concentrations. After treatment, mean egg testosterone concentrations of different families ranged from 0.20 to 0.59 ng.egg^{-1} as compared to the control range of 0.02-0.16 ng.egg^{-1}. The treated levels could therefore be said to be in the high physiological range.

Table 2.1. Maternal and egg parameters for each family. Gonadosomatic index is estimated by 100 x (weight loss through stripping/pre-stripped weight). Where appropriate, mean values ± SE are provided. Weight gain through hydration is calculated as the difference between mean egg weights before and immediately after treatment. % Weight increase is calculated as 100 x (weight gain/unfertilised weight). Mean steroid concentrations in control and steroid-treated eggs are from immediately after treatment (n=6 for each sampling point). Steroid uptake is calculated as the difference between control and treated concentration means. % Increase is calculated by 100 x (uptake/control).

	Family 1	Family 2	Family 3	Family 4	Family 5
Female fork length (cm)	31.1	34.2	31.2	30.6	30.7
Female weight (g)	345	497	362	326	354
Weight loss through stripping (g)	59.3	79.8	55.7	49.4	64.2
Gonadosomatic Index (%)	17.2	16.1	15.4	15.2	18.2
Plasma cortisol ($ng.ml^{-1}$)	8.02	25.04	20.19	50.45	45.67
Plasma testosterone ($ng.ml^{-1}$)	0.87	0.96	< 0.20	4.25	5.92
Ovarian fluid cortisol ($ng.ml^{-1}$)	6.08	8.48	6.04	14.38	15.09
Ovarian fluid testosterone ($ng.ml^{-1}$)	< 0.05	0.41	0.24	0.44	2.07
Unfertilised weight (mg)	51.1 ± 0.7	49.1 ± 0.6	48.6 ± 0.9	49.7 ± 0.5	50.5 ± 0.6
Post-treatment weight (mg)	60.5 ± 0.5	58.8 ± 0.6	57.0 ± 0.7	57.6 ± 0.7	61.3 ± 0.4
Weight gain through hydration (mg)	9.4	9.7	8.3	7.9	10.8
% Weight increase	18.2	19.9	17.1	16.0	21.4
Control cortisol ($ng.egg^{-1}$)	0.19 ± 0.01	0.17 ± 0.01	0.10 ± 0.01	0.25 ± 0.02	0.12 ± 0.01
Treated cortisol ($ng.egg^{-1}$)	0.63 ± 0.04	0.47 ± 0.09	0.59 ± 0.02	0.82 ± 0.07	1.10 ± 0.03
Uptake (ng)	0.44	0.30	0.49	0.57	0.98
% Increase	18	29	40	32	183
Control testosterone ($ng.egg^{-1}$)	0.07 ± 0.00	0.16 ± 0.01	0.12 ± 0.01	0.02 ± 0.00	0.13 ± 0.00
Treated testosterone ($ng.egg^{-1}$)	0.30 ± 0.01	0.34 ± 0.01	0.59 ± 0.03	0.20 ± 0.01	0.21 ± 0.02
Uptake (ng)	0.23	0.18	0.47	0.18	0.08
% Increase	329	113	392	900	62

Figure 2.1. Mean hormone concentrations in control and treated eggs. Time axis is not to scale; U - unfertilised eggs, h - hours post-fertilisation, d - days post-fertilisation. F: cortisol, T: testosterone. Error bars represent ± SE.



Immediately following cortisol-treatment, cortisol concentrations in eggs from all families far exceeded control values. However, by two days post-treatment, the treated range (0.11-0.30) overlapped the control range for the five families (0.06-0.25). There was no association between the amount of steroid and amount of water taken up by the egg during treatment.

Resting metabolic rate

The weight-corrected resting metabolic rate data (hereafter, rMR data) were analysed using a two-way ANOVA, again with Family and Treatment as fixed factors. As was the case with the yolk-utilisation data, there were significant factor and interaction effects, and the factors were therefore examined separately using one-way ANOVA and Tukey's *post hoc* comparisons (Tables 2.3. & 2.4.). Significant treatment effects within families were only apparent in family 4, where cortisol and testosterone treatments were associated with a reduction in the mean metabolic rate compared with control levels. Control individuals from family 4 had a significantly higher mean rMR than control fish from family 3. These differences between families were abolished by testosterone treatment, and somewhat reversed by cortisol treatment.

Juvenile size and condition factor

Size and condition factor data were analysed for family and treatment effects using two-way ANOVA. In each case, there were significant interaction effects, and the data were therefore analysed separately by each factor.

Family differences in size and condition factor are summarised in Table 2.4. In general, individuals from family 1 were larger than fish from the other families, and fish from family 3 were smaller. Testosterone and cortisol treatment affected the magnitude of these differences, but general trends of inter-family differences were similar in the three treatment groups. Significant effects of treatment were found within three families, although, once again, there were no consistent trends (Table 2.3.). In family 3, testosterone treatment significantly increased length, weight, and condition factor over control levels. In families 4 and 5, cortisol treatment increased size and condition factor compared with the level of controls and testosterone-treated fish.

Table 2.2. Mean values (± SE) for physiological variables measured. rMR: residual resting metabolic rate (n=20/group). Condition factor estimated by 100(weight/length^3). Size, condition factor and sex ratio data are derived from n≥40/group. F: cortisol, T: testosterone, N/S: non-significant. One-way ANOVA and Tukey's post-hoc tests with a family error rate of 0.05.

	Family 1			Family 2			Family 3		
	Control	F	T	Control	F	T	Control	F	T
rMR (ml $O_2.h^{-1}$ x10^{-3})	19.3 ± 22.6	9.8 ± 12.4	24.6 ± 14.0	81.1 ± 30.1	163.5 ± 41.5	48.3 ± 49.5	-19.1 ± 19.0	6.1 ± 40.9	-13.3 ± 24.9
Final weight (g)	6.5 ± 0.4	6.3 ± 0.2	6.1 ± 0.3	5.4 ± 0.2	5.8 ± 0.2	5.9 ± 0.2	3.3 ± 0.1	3.7 ± 0.2	3.9 ± 0.1
Final length (cm)	8.12 ± 0.13	8.11 ± 0.09	7.95 ± 0.09	7.70 ± 0.09	7.89 ± 0.07	7.96 ± 0.08	6.70 ± 0.09	6.86 ± 0.10	7.05 ± 0.08
Condition factor	1.16 ± 0.01	1.15 ± 0.01	1.15 ± 0.01	1.15 ± 0.01	1.14 ± 0.01	1.15 ± 0.01	1.06 ± 0.01	1.07 ± 0.01	1.09 ± 0.01
Sex ratio (F:M)	0.79	0.62	0.95	1.17	0.92	0.78	0.50	1.05	1.03

	Family 4			Family 5		
	Control	F	T	Control	F	T
rMR (ml $O_2.h^{-1}$ x10^{-3})	188.4 ± 58.9	-36.5 ± 37.1	8.7 ± 33.9	74.9 ± 55.3	68.5 ± 46.4	-39.5 ± 43.9
Final weight (g)	5.0 ± 0.2	6.0 ± 0.2	5.1 ± 0.2	5.0 ± 0.2	6.2 ± 0.3	5.2 ± 0.2
Final length (cm)	7.55 ± 0.10	7.94 ± 0.10	7.60 ± 0.11	7.68 ± 0.08	8.15 ± 0.12	7.73 ± 0.10
Condition factor	1.14 ± 0.01	1.15 ± 0.01	1.11 ± 0.01	1.08 ± 0.01	1.12 ± 0.01	1.09 ± 0.01
Sex ratio (F:M)	0.96	1.23	0.83	0.91	0.83	0.42

Table 2.3. Results of within-family one-way ANOVA of physiological variables by treatment group. :rMR: residual resting metabolic rate (n=20/group). Condition factor estimated by 100(weight/length^3). Size and condition factor data are derived from n≥40/group. F: cortisol, T: testosterone, N/S: non-significant. Tukey's post-hoc tests with a family error rate of 0.05. '*' indicates that dominance rank is significantly affected by individual weight, with larger individuals being more likely to be dominant ($p<0.05$).

	Family 1	Family 2	Family 3	Family 4	Family 5
rMR (ml $O_2.h^{-1}$ x10^{-3})	N/S	N/S	N/S	Control>F,T	N/S
Final weight (g)	N/S	N/S	T>Control	F>Control,T	F>Control,T
Final length (cm)	N/S	N/S	T>Control	F>Control	F>Control,T
Condition factor	N/S	N/S	T>Control	F>T	F>Control,T
Dominance	T>Control*	N/S*	N/S*	N/S	N/S

The final size of control fish from each family was not correlated with either unfertilised or hydrated egg weight.

Table 2.4. Results of within-treatment one-way ANOVA of physiological variables by family. See legend for Table 3 for details of variables. N/S: non-significant. Numbers in table refer to families.

	Control	Cortisol	Testosterone
rMR (ml $O_2.h^{-1}$ x10^{-3})	4>3	2>1,4	N/S
Final weight (g)	1>3,4,5 2,4,5>3	1,2,4,5>3	1,2,4,5>3 1,2>4
Final length (cm)	1>2,4,5>3	1,2,4,5>3	1,2,4,5>3
Condition factor	1,2,4>3,5	1,2,4,5>3	1,2>3,4,5

Sex ratios

The ratio of females to males for each treatment in each family is shown in Table 2.2. Sex data were analysed using a generalised linear model with binary error structure, with Treatment as a fixed effect and Family as a random effect. There was no effect of treatment on sex ratios ($p>0.05$).

Effects of hormone treatment on behavioural dominance

Fish were ranked within each triad (1 = 'dominant'), and individual weight was transformed to a proportion of the triad mean weight. A polytomous logistic regression model (Hosmer & Lemeshow 1989) was then used to examine the relationship between treatment, dominance rank and proportional weight. When data from all families were combined, the treatment effect was non-significant ($\chi^2=0.5734$, $p=0.6161$), but weight was highly significant ($p<0.001$), with the largest fish being more dominant. Given the high degree of variation between families in response to treatment, it seemed sensible to conduct analyses of dominance data for each family also. Treatment significantly affected dominance in family 1 only, where testosterone-treated fish were significantly more likely to be dominant than control fish (Table 2.3.). Weight significantly affected dominance rank in families 1-3, with larger fish being significantly more likely to be dominant (Table 2.3.). Expressed as a proportion of the mean weight for the triad, the 95 % confidence interval for variation about the triad mean was 1 ± 0.0082. So, in most cases, weight differences within triads were very small.

Relationships between treatment effects

Final juvenile size and condition factor measures differed between treatment groups in three of the five families tested, and hormone treatment also reduced the metabolic rate of individuals in one of these families. In two families, offspring from a particular treatment group were significantly larger than the controls when the final juvenile size measures were taken. This response was found in the testosterone-treated offspring of family 3, and in the cortisol-treated offspring of family 4. In family 5, testosterone-treated offspring were smaller than cortisol-treated fish at the end of the experiment. Compared with the controls, cortisol-treated offspring of family 4 were larger at the end of the experiment, and they also had significantly lower metabolic rates. Testosterone-treated individuals in family 4 also had a lower mean metabolic rate than controls, but there was no significant difference in the size of testosterone-treated and control fish at the end of the experiment.

Discussion

Individual variation in the physiology and behaviour of juvenile salmonids can have significant impacts on life history patterns, but the factors contributing to the development of this individual variation are not well understood (Marschall *et al.* 1998, Metcalfe 1998, Thorpe *et al.* 1998, Sloman & Armstrong 2002). Recent studies on tropical damselfish have indicated that larval and juvenile size variation may be related to levels of maternal cortisol and testosterone deposited in eggs prior to spawning (McCormick 1998, 1999). My experiment was designed to examine whether variation in egg testosterone and cortisol concentrations could contribute to variation in the physiology and behaviour of juvenile brown trout. To the best of my knowledge, this is the first experiment to examine directly the effects of initial egg testosterone concentrations on aspects of development in salmonids other than sexual differentiation. It is also the first to examine, in any species of fish, the effects of egg hormone levels on juvenile behaviour and metabolic rate.

I artificially elevated concentrations of testosterone or cortisol in five families of brown trout eggs and then compared juvenile sex ratios, size, condition, metabolic rate and dominance behaviours of control and treated progeny of the same family. Within each family, mean physiological and behavioural variables could vary between fish

from different treatment groups. However, these differences were not consistent between families, raising the question of whether the observed differences in offspring physiology and behaviour were a consequence of steroid treatment, or whether they were artefacts of the experimental design.

Slight variations in environmental conditions (e.g., lighting, turbulence) can affect the performance of juvenile fish. In fish biology, such effects are generally known as 'tank effects.' If the effects of an experimental treatment are profound and consistent, then tank effects may not be a factor. In a case like this one, where differences between groups were subtle and variable, determining whether the differences were treatment-related becomes difficult. Replication of each treatment within each family may have allowed me to determine whether the effects were definitely treatment-related (in which case there would have been consistent effects within intra-family replicates of each treatment), but this was not logistically possible. Instead, every attempt was made to ensure that fish from each group were reared under identical conditions (see methods), and I also checked that there was no systematic relationship between the distribution of effects and the rearing position of the groups. For example, fish from groups reared in tanks at one end of the hatchery were not larger than fish from the same families reared in other areas. However, it remains unclear as to whether the effects recorded here are a real response to manipulation of egg steroid content, or to undetected variation in rearing conditions.

Inherent variability between families

There were no differences in egg weight between control offspring of the different families, but there were significant differences between families in juvenile metabolic rate and size at the end of the experiment. Eggs from all families were fertilised with milt pooled from the same three males. Assuming that eggs from each family received the same parental gamete mix, the physiological variation found between control groups is probably due to maternal effects, both genetic and non-genetic. However, once again, tank effects cannot be ruled out.

Family variation in egg composition and hydration

In terms of non-genetic effects, this experiment has shown that the degree of post-spawning egg hydration, and egg testosterone levels, vary between families. Egg

mortality rates were very low in all groups. The degree of egg hydration following fertilisation appeared to be related to the total weight of gametes relative to maternal body weight (gonadosomatic index, I_G), but was not related to female size. That is, eggs from females with greater relative fecundity (eggs.kg body mass^{-1}) hydrated more than eggs from females with lower relative fecundity. While my intra-family analyses are highly robust, results from between-family regression and correlation analyses (such as that between I_G and egg hydration) should be regarded with some caution given that only five families could be sampled.

There was considerable variation among families in the concentrations of testosterone, but not cortisol, in untreated eggs. The main function of testosterone in the developing ovary is to act as a precursor in the synthesis of 17β-estradiol, the primary estrogen involved in female teleost reproduction. Cortisol, meanwhile, is elevated in the plasma during the final stages of ovarian development, but why this is, and whether cortisol plays a role in ovarian development, is not well understood (see reviews by Leatherland 1999, Schreck *et al.* 2001).

Effect of treatment on egg steroid concentrations

Immersion of eggs in water baths containing steroids during the period of water hardening caused an elevation of steroid concentrations in all families. The amount of steroid absorbed varied between families, but did not appear to be related to initial egg hormone concentrations, initial egg size, or to degree of egg hydration. There was no significant change in hormone concentrations in control or testosterone-treated eggs at two or seven days post-treatment, but 57-84 % of the cortisol measured in cortisol-treated eggs immediately after treatment was not detected in the eggs two days later.

A similar post-treatment reduction in immunoreactive cortisol has been reported in other immersion experiments (Brooks *et al.* 1995; Stratholt *et al.* 1997). Stratholt *et al.* (1997) reported that the clearance of cortisol from the eggs was such that, by eight days post-fertilisation, there were no significant differences between treated and control egg cortisol concentrations. While it is possible that cortisol is 'washed out' of the eggs after waterhardening, it is also possible that cortisol is metabolised within the yolk sac to forms that are not detectable by the radioimmunoassay used here. For

example, steroids are commonly sulphated or glucuronated within the system of adult fish (Yeoh *et al.* 1996a), and concentrations of these conjugated forms of cortisol would not have been measured by my assay system. Several studies (Pillai *et al* 1974, Antila 1984, Yeoh *et al.* 1996a,b, Khan *et al.* 1997a,b,c) have demonstrated that embryos are capable of metabolising exogenous steroids very early in development. It is also possible that maternal materials deposited in the yolk-sac include enzymes capable of metabolising maternal steroids. Thus, although not detected by the assay system used here, it is possible that large amounts of conjugated cortisol or cortisol metabolites were retained in the eggs, and could play some role in embryonic development.

In this study, testosterone concentrations did not change significantly between fertilisation and seven days post-fertilisation. This result contrasts with those of Feist & Schreck (1996), who found that *c.* 15 % of the testosterone present in unfertilised rainbow trout eggs was undetectable by five days post-fertilisation, and 60 % undetectable by 10 days post-fertilisation. In coho salmon eggs, only *c.* 10 % of the testosterone present at fertilisation was measured at 10 days post-fertilisation (Feist *et al.* 1990). Inter-species variation in the rates of testosterone metabolism or clearance following fertilisation could be due to species-related differences in egg surface area, in the permeability of the egg membrane to testosterone following fertilisation and water-hardening, or in the onset of metabolism and clearance by the developing embryo. Alternatively, significant metabolism or clearance of testosterone from brown trout eggs may occur more than seven days post-fertilisation.

By two days post-treatment, steroid concentrations in most treatment groups were within the physiological range, to the extent that some treated egg steroid concentrations were lower than control steroid concentrations in other families.

Effects of treatment on offspring physiology

I am unable to determine whether the effects reported here are due to treatment or tank effects in the absence of further replication within each female. If the differences observed in this experiment are due to manipulation of egg steroid content, then cortisol, testosterone, or their metabolites are either acting directly upon the developing embryo, or with other materials within the egg to affect the course of development.

These materials could be metabolising enzymes, binding proteins, hormone receptors, or other hormones to name just a few possibilities. An increase in egg cortisol or testosterone content as a result of treatment could modify these actions. If the steroids or their metabolites are interacting with other materials within the egg, and amounts of these other compounds varied between eggs of different females, then the inter-family variation in treatment effects could be explained.

Sexual differentiation and egg steroid concentrations

The process of sexual differentiation in salmonids seems to start around hatching, and eventual phenotypic gender can be manipulated by exposing eyed eggs and newly hatched alevins to androgens (e.g., Piferrer *et al.* 1993; Feist *et al.* 1995). Testosterone treatment had no affect on sex ratios in this study, possibly because the steroid elevation was insufficient to cause sex reversal, or perhaps because testosterone concentrations in treated eggs may have declined to control levels prior to the testosterone-sensitive stage of sexual differentiation. Sex steroid concentrations in developing embryos of coho salmon and rainbow trout decline sharply after fertilisation and remain at very low levels until rising again around hatching (Feist *et al.* 1990; Feist & Schreck 1996). The increase in embryonic sex steroid concentrations around hatching possibly induces sexual differentiation (Feist & Schreck 1996), which would explain the sensitivity of developing fish to environmental androgens at this time. If testosterone content in testosterone-treated and control eggs did decline to the same level prior to hatching, and if the hatching rise of sex steroid concentrations and subsequent sexual and gonadal differentiation were unaffected by original testosterone concentrations in the fertilised egg, then I would expect no difference in offspring sex ratios. Unfortunately, testosterone content of fish at hatching was not measured, so this comparison between controls and treated offspring could not be made.

A possible relationship between egg cortisol levels and sex differentiation has not, to the best of my knowledge, been examined previously. There was no apparent effect of cortisol treatment on sex ratios in this study.

Egg steroid concentrations, juvenile size and juvenile dominance

Although there was no overall effect of steroid treatment on the likelihood of acquiring dominance, separate analysis of each family suggested that testosterone-treated

progeny from one female may have been competitively advantaged over their siblings. This could again be due to a tank effect. Relative fish size had significant effects on the outcome of the behavioural trials, with larger individuals being more likely to be dominant than smaller siblings. This finding is consistent with previous studies on factors determining dominance in juvenile salmonids under similar conditions (see review by Sloman & Armstrong 2002).

Ecological implications

In the absence of a clear answer as to whether the effects measured in this experiment can be attributed to treatment, it is premature to speculate on the ecological importance of maternal steroids in eggs. If the effects are the result of treatment, then the potential exists for concentrations of maternal steroids in eggs to affect ecologically important traits such as metabolic rate and juvenile size. Further study is required before the role of maternal steroids in the egg at fertilisation can be confidently assessed.

CHAPTER THREE

INTRA-FEMALE VARIATION IN BROWN TROUT FOLLICLE SIZE AND CORTISOL CONTENT

Abstract

This study investigated the extent of intra-female variation in follicle size and cortisol levels in seven mature pre-ovulatory brown trout, and in egg size and cortisol content in four post-ovulatory brown trout. Follicle weight and cortisol content differed between fish and between ovary regions (top, bottom, or centre of the ovaries as they lie in the body cavity), but did not differ significantly between left and right ovaries. Regional differences in follicle weight and cortisol content were not consistent between fish. Follicle weight and cortisol content were positively related across females, but were not associated within females. While there were no regional differences in egg weight in fish that had ovulated (eggs in an unbound mass in the body cavity), egg cortisol content was significantly higher in the anterior region of the body cavity. The amount of follicle/egg variability differed between females, but not between regions within a female. Coefficients of variation (CVs) for weight were much lower than CVs for cortisol content. CV ranges were similar for pre-ovulatory and ovulated females. I hypothesise that intra-female and intra-ovary variation in follicle weight and cortisol content may be explained by proximity to blood vessels. These results demonstrate that there is variation in the weight and cortisol content of gametes of a single female, providing a possible explanation for the individual variation found within full-sibling salmonid families.

Introduction

Individuals within both wild and cultured salmonid populations vary greatly in aspects of their physiology and behaviour that are known to impact on life history strategies (Metcalfe 1998). For example, standard metabolic rate at the onset of exogenous feeding has been linked with aggressiveness (Metcalfe *et al.* 1995, Cutts *et al.* 1999a), aggressiveness linked with juvenile growth (Huntingford *et al.* 1990, Sloman *et al.* 2001b), and juvenile growth linked with age at maturation and fecundity (Thorpe *et al.* 1984, Jonsson *et al.* 1996).

Individual variation in such ecologically important traits is present within full-sibling groups of fish (Metcalfe *et al.* 1995, Garcia de Leaniz *et al.* 2000). While some of this will be due to genetic variation, the genetic basis of individual variation in a group of full-siblings is much reduced when compared to the population as a whole, and the

effects of early environment on individual development are likely to become more apparent (see Chapter 6). In the wild, early environmental variation might include the position of individual eggs within a redd (the gravel-enclosed nest in which the eggs develop), which may influence oxygen availability. In a hatchery situation, the early environment will be fairly uniform, and it might be supposed that variation in hatchery-reared juveniles could therefore be attributed to genetic variation. However, the egg itself is an important part of the environment of a developing fish, and variation in characteristics of the egg could result in variation in the development, physiology, and behaviour of offspring.

Two areas of egg variability that have received attention are egg size and composition. Egg size correlates with larva size at hatching and emergence both between (Glebe *et al.* 1979, Kazakov 1981, Thorpe *et al.* 1984) and within full-sibling groups (i.e., families) of salmonids (Einum & Fleming 1999). Post-emergent size differences are generally short-lived in the hatchery environment (Thorpe *et al.* 1984, Springate & Bromage 1985, Einum & Fleming 1999), but it is possible that they are maintained under more natural conditions (Einum & Fleming 1999). Variation in salmonid egg size within a family may therefore lead to individual variation in juvenile size under certain conditions. To the best of my knowledge, the effects of variation in egg size on aspects of behaviour or physiology other than growth and survival have not been examined in full-siblings.

Environmental experiences during ovarian development can result in variation in the composition of eggs from different females, and can affect gamete quality, and offspring survival and development (see review by Brooks *et al.* 1997). Whether these factors also affect the physiology and behaviour of juvenile salmonids is seldom studied. Unfertilised salmonid eggs contain large amounts of hormones (e.g., Tagawa & Hirano 1987, Feist *et al.* 1990, Leatherland 1999). It has been hypothesised that these hormones might play a role in early embryonic development, regulating developmental processes prior to endogenous hormone production (Lam 1994, Brooks *et al.* 1997). In Chapter 2 I have considered whether cortisol and testosterone concentrations in brown trout (*Salmo trutta* L.) eggs may influence juvenile metabolic rate, growth and behaviour, but have not considered whether egg hormone levels vary

within a female. Variation in egg hormone levels within a female may generate variation between full-sibling individuals.

This study examines the extent of intra-female variation in egg size and egg cortisol concentrations, using hatchery-reared brown trout. I investigated levels of cortisol because it is generally present in quite high quantities in brown trout eggs (Chapter 2). Both vitellogenin and cortisol are transported to the ovaries in the bloodstream. If uptake of these molecules by oocytes is related to proximity to blood vessels, and there is variation in the proximity of individual oocytes to blood vessels, then there is scope for variation in oocyte size and cortisol content within a single female.

To ensure that the data collected in this study would be relevant to a natural spawning situation, I investigated whether egg size or cortisol content varied depending on developmental position within the ovary and body cavity. Naturally-spawning salmonid females generally deposit eggs in more than one nest over a period of hours or days (Fleming 1998), and eggs deposited in the first nest come from the rear of the body cavity (Chapter 4). If there are significant differences in the weight and/or cortisol content of eggs from different regions of the body cavity, then I can hypothesise a potential mechanism for the development of physiological and behavioural variation in full-siblings from different nests. Alternatively, if variation in egg size and cortisol content is similar throughout the ovaries, then I can hypothesise a potential mechanism for the development of intra-nest variation.

I examined variation in egg size and cortisol content just prior to ovulation. That is, the oocytes had finished their growth phase, but were still encapsulated by a follicle layer and the ovaries were intact. It was not practical to remove ova from these follicle layers, so I assessed follicle weight and follicular cortisol content. The ovulation (breakdown of the ovaries and expulsion of oocytes from the follicle layer into the body cavity) of four females prior to sampling allowed me to contrast gamete size and cortisol content data from pre-ovulatory and ovulated females.

This study specifically addressed the following questions:

1 What is the extent of intra-female variation in follicle weight and follicular cortisol content just before spawning?

2 What is the extent of intra-female variation in ovulated egg weight and egg cortisol content just before spawning?

3 Is this variation related to position within the body cavity?

4 How does cortisol content relate to follicle/egg weight?

Methods

Hatchery-reared 2 year old virgin (1st time matured) female brown trout (mean forklength ± SE = 32.6 ± 0.5 cm, n=11) were purchased from Howietoun Fishery (Stirling) in late October and transferred to holding tanks at the University of Glasgow. The females had not ovulated at the time of arrival at Glasgow. In the three days following their arrival, seven pre-ovulatory and four ovulated females were terminally anaesthetised with benzocaine (0.1 $g.l^{-1}$) and blood-sampled. Blood samples were taken from the caudal vein, centrifuged at 3000g for 5 min, and the plasma collected and frozen at –20 °C for hormone analyses. A sample of ovarian fluid was also taken from each ovulated female.

The fish were dissected along the ventral mid-line, from the anus to the pectoral girdle. The position of the ovaries within the body cavity, and the position of blood vessels in relation to the ovaries, was recorded. The ovaries were then dissected out and weighed, before being transferred to petri dishes and held on ice.

Each ovary was examined for regional differences in follicle weight, stage of development, and cortisol levels. Approximately 100 follicles from the anterior ('top'), centre and posterior ('bottom') of each ovary were teased apart from ovarian connective tissue. At least six samples of follicles (each having a total weight of > 0.2 g; 3-5 follicles depending on follicle size) were then taken from each region for steroid analyses. The follicles were blotted dry, individually weighed and transferred to Eppendorf tubes to be frozen at –20 °C. Thus at least 18 follicles from each of the six ovary regions were individually weighed for each female. Due to the size of the follicles and sensitivity limits of the cortisol assay (see Appendix 2), individual

follicles could not be used for separate steroid analyses. In the case of the ovulated females, eggs were collected from the top, centre and bottom of the body cavity, and treated as for the follicles from intact females. Unfortunately, data on individual egg weight were not collected for one of the ovulated females, although egg cortisol data was collected for this individual.

A preliminary study measured follicle diameter in addition to follicle weight. As the follicles were not uniformly round and the process of separating the follicles from connective tissue could result in changes in follicle shape, follicle weight data were considered the more robust measure of follicle size, and diameters were not measured in the larger study. There was a direct relationship between the diameter at the widest point of the follicle and weight, however, with diameter increasing with follicle weight (regression: F=61.84, p<0.001, n=167).

To stage oocyte development, a clearing solution (0.9% NaCl:glacial acetic acid:glycerol at 30:3:2) was used to visualise the position of the germinal vesicle. The germinal vesicle migrates to the cell periphery just prior to the resumption of meiosis and ovulation (Wallace & Selman 1981). Follicles were placed in a petri dish, covered with clearing solution, and viewed under a light microscope with sub-stage illumination.

Steroid analyses

The extraction procedure was standardised to reduce possible extraction errors (see Appendix 1). Each follicle sample was thawed at room temperature, and the follicle layer and oocyte membranes disrupted by hand using 20 strikes of a mounted needle. Sodium hydroxide (200 µl of 0.1M) was then added to each Eppendorf tube, and the samples vortex mixed for 30 s. After 1 h at room temperature, 1.0 ml of ethyl acetate (AR grade) was added to each Eppendorf tube. The tubes were capped and vortex mixed for 30 s, then spun at 13000 rpm for 5 min to separate the phases. 0.9 ml of the upper, solvent, phase was transferred into a clean, labelled Eppendorf tube, and a further 0.5 ml of ethyl acetate was added to the sample Eppendorf tube. After vortex mixing, the sample was again centrifuged, and 0.56 ml of solvent was removed into the same extract Eppendorf tube. Eppendorf tubes were capped between extractions to

prevent solvent evaporation. Steroids in the ovulated egg samples were extracted using the same method. Plasma cortisol was extracted by transferring a known volume of plasma to a clean Eppendorf tube and adding solvent as described above. Extracts were stored upright in sealed containers at –20 °C until assayed.

Extract cortisol concentrations were measured using radioimmunoassay, following the method of Pottinger & Carrick (2001) (see Appendix 2). Extraction efficiency was assessed by measuring recovery of tritiated cortisol from samples as described in Appendix 1. The mean ± SE % recovery of cortisol 59.5 ± 0.5 % and 97.6 ± 0.3 % (n=6) for follicle tissue and plasma, respectively. Concentrations are expressed as pg.follicle^{-1}, pg.egg^{-} or ng.ml^{-1} plasma, and are not corrected for recovery. Serial dilutions of plasma and gamete extracts showed that the dilution curves were parallel to the standard curve over the extract volumes used.

Statistical analyses

Asymmetries in ovary weight were examined using a paired t-test. Follicle weights and follicle cortisol concentrations of pre-ovulatory females were analysed using three-way ANOVA with fish (7 levels), ovary (2 levels) and ovary region (3 levels) as factors. Egg weights and egg cortisol concentrations of ovulated females were analysed using two-way ANOVA with fish (3-4 levels) and body cavity region (3 levels) as factors. Coefficients of Variation (CVs) were calculated as the standard deviation divided by the mean, and expressed as a percentage. CVs were also examined using ANOVAs. Associations between variables were examined using correlation. A p-value of less than 0.05 was considered significant. For graphical purposes, and because of significant interaction between factors, regional differences in follicle weight and cortisol concentration within each family were examined using one-way ANOVA. Data were transformed for normality if necessary.

Results

Pre-ovulatory females

All pre-ovulatory females had asymmetric ovaries (Table 3.1.). In each case, the fish's right ovary was much shorter and smaller than the left (paired t-test, H_a: right ovary < left ovary, n=7, t=-4.20, p=0.003). Large blood vessels passed longitudinally along the

dorsal surface of the ovaries, from the anterior end, to approximately two-thirds of the ovary length where it subdivided into smaller vessels. Smaller lateral vessels branched off along the entire length of the ovary (Figure 3.1.), and were further subdivided into a network of narrow blood vessels passing throughout the ovarian tissue. All fish contained oocytes in which the germinal vesicle had started to migrate toward the cell periphery, indicating that the oocytes were very close to the resumption of meiosis. This advanced stage of maturation was further confirmed by the ovulation of some of the females before I was able to process them.

Figure 3.1. Freshly dissected brown trout ovaries, showing some of the associated blood vessels.

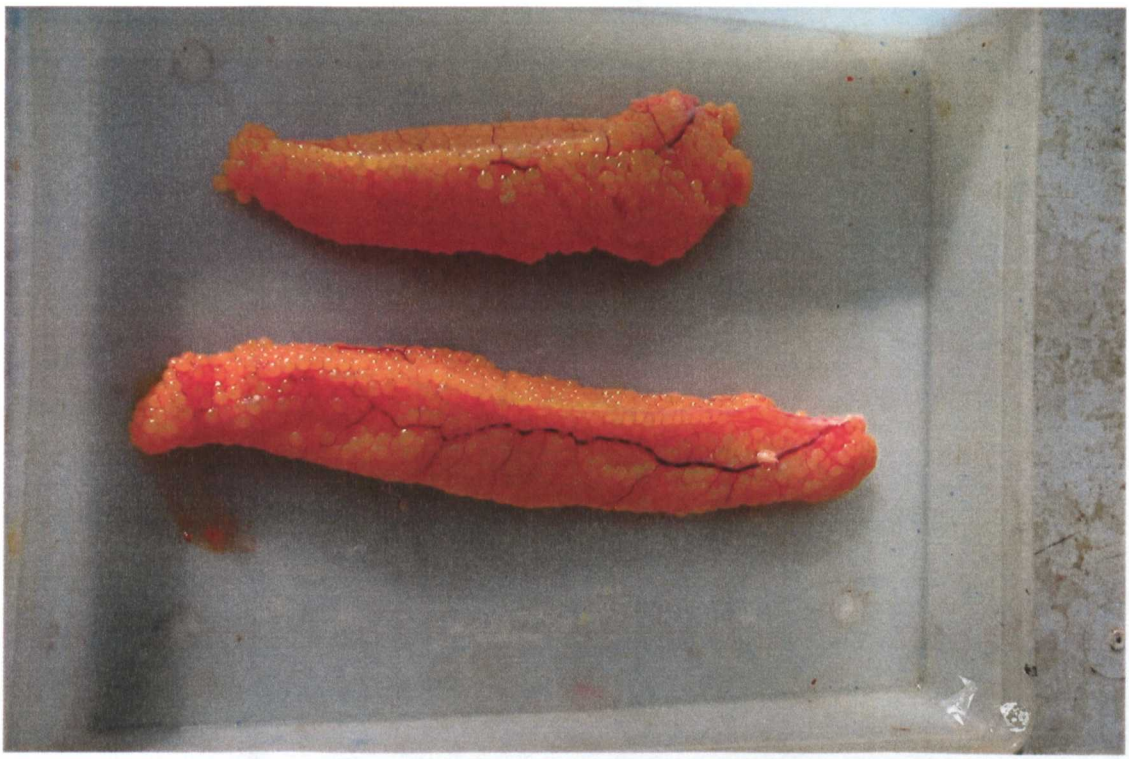


Table 3.1. Ovary weights of pre-ovulatory females used in this study.

	Female						
	A	B	C	D	E	F	G
Right ovary (g)	39.2	46.2	16.4	25.4	32.4	14.3	41.7
Left ovary (g)	49.0	60.9	34.7	43.7	38.6	43.3	44.2

Table 3.2. Follicle weight and cortisol (F) content data for the pre-ovulatory study animals. Mean values are given ± SE.

	Female						
	A	B	C	D	E	F	G
Mean follicle weight (mg)	59.9 ± 0.2	51.4 ± 0.1	69.1 ± 0.2	46.2 ± 0.2	47.6 ± 0.2	51.8 ± 0.2	60.9 ± 0.2
CV (%)	4.97	3.87	2.97	5.35	4.42	5.94	4.39
Mean follicle F content (pg)	188 ± 4	102 ± 5	456 ± 11	59 ± 2	141 ± 3	137 ± 4	170 ± 3
CV (%)	13.08	27.48	13.09	21.12	11.82	16.04	9.38
Plasma F (ng ml^{-1})	1.72	1.97	1.67	0.52	3.33	2.10	2.93

The coefficient of variation (CV) of follicle weight ranged from 2.97 – 5.94 % (Table 3.2.). There was no association between mean follicle weight and CV (correlation, r= -0.601, p>0.05). Within female variation in follicular cortisol content was greater, with CVs ranging from 9.38 – 27.48 % (Table 3.2.). There was no association between mean follicular cortisol content and CV (r=-0.575, p>0.05), or between mean follicular cortisol content and maternal plasma cortisol concentrations (r=0.462, p>0.05). There was no association between weight CVs and cortisol content CVs (r=0.091, p>0.05), but means of follicular weight and cortisol content were positively associated (r=0.890, p<0.01).

Maternity and ovary region had significant effects on follicle weight, but whether follicles were from the left or the right ovary had no effect on follicle weight. Interaction factors of the ANOVA model reflect a general lack of consistency in patterns of follicle weight between females and ovaries (Table 3.3.). The same effects and inconsistencies were evident when the cortisol content (pg.follicle^{-1}) of follicles were analysed (Table 3.3.). Mean weight and cortisol content for different regions are plotted for each pre-ovulatory female (Figures 3.2. & 3.3.). There appears to be a general trend for follicles from the centre of the ovaries to be heavier than those from the top or bottom regions.

Table 3.3. Results of 3-way ANOVA of follicle weight and cortisol content data from seven pre-ovulatory females.

	Follicle weight			Cortisol (pg follicle^{-1})		
	ν	*F*	*p*	ν	*F*	*p*
Fish	6	2153.70	<0.001	6	1354.5	<0.001
Ovary	1	1.06	0.304	1	2.55	0.112
Region	2	19.73	<0.001	2	4.08	0.018
Fish x Ovary	6	4.00	0.001	6	35.76	<0.001
Fish x Region	12	3.80	<0.001	12	2.20	0.013
Ovary x Region	2	9.65	<0.001	2	3.61	0.029
Fish x Ovary x Region	12	2.89	0.001	12	3.33	<0.001
Error	1182			178		
Total	1223			219		

When the mean regional weight and cortisol content data for all seven pre-ovulatory females are plotted against each other (Figure 3.4.), it is evident that cortisol content increases with increased follicle weight between females (Spearman's rank correlation,

r_s=0.855, p<0.001), but that this relationship does not hold within females (Pearson's correlations for each female, p>0.05, -0.613 ≤ r ≤ 0.452). The scatter of within-family means in Figure 3.4., and the wide range of correlation coefficients illustrates the lack of any consistent relationship between follicle size and cortisol content.

Figure 3.2. Regional variation in follicle weight. Each graph represents follicles from a different female (maternity indicated by letter). Right ovary shaded. Columns represent the mean + SE. Columns marked with different letters are significantly different (One-way ANOVA and Tukey's post-hoc tests. p<0.05).

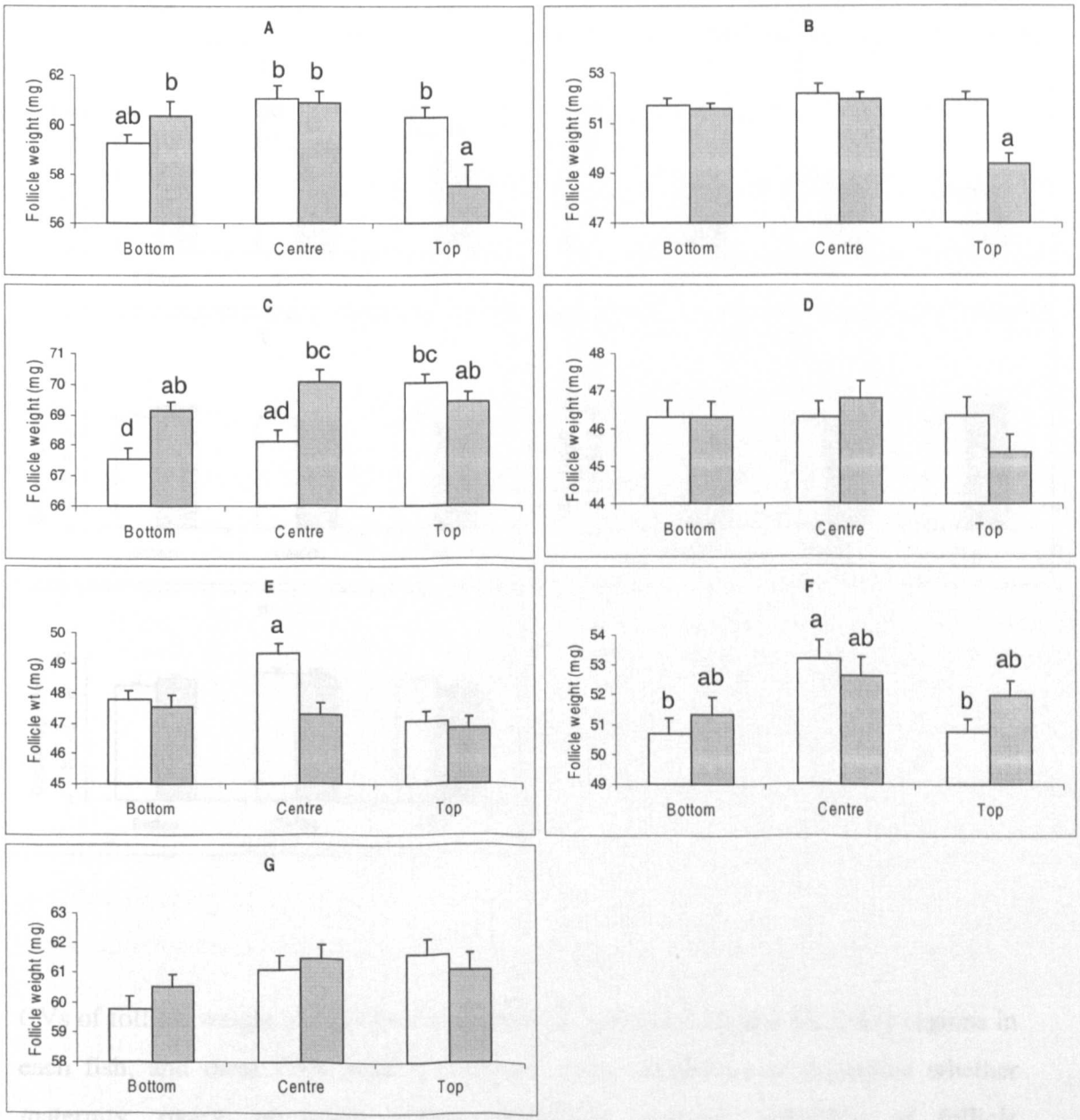
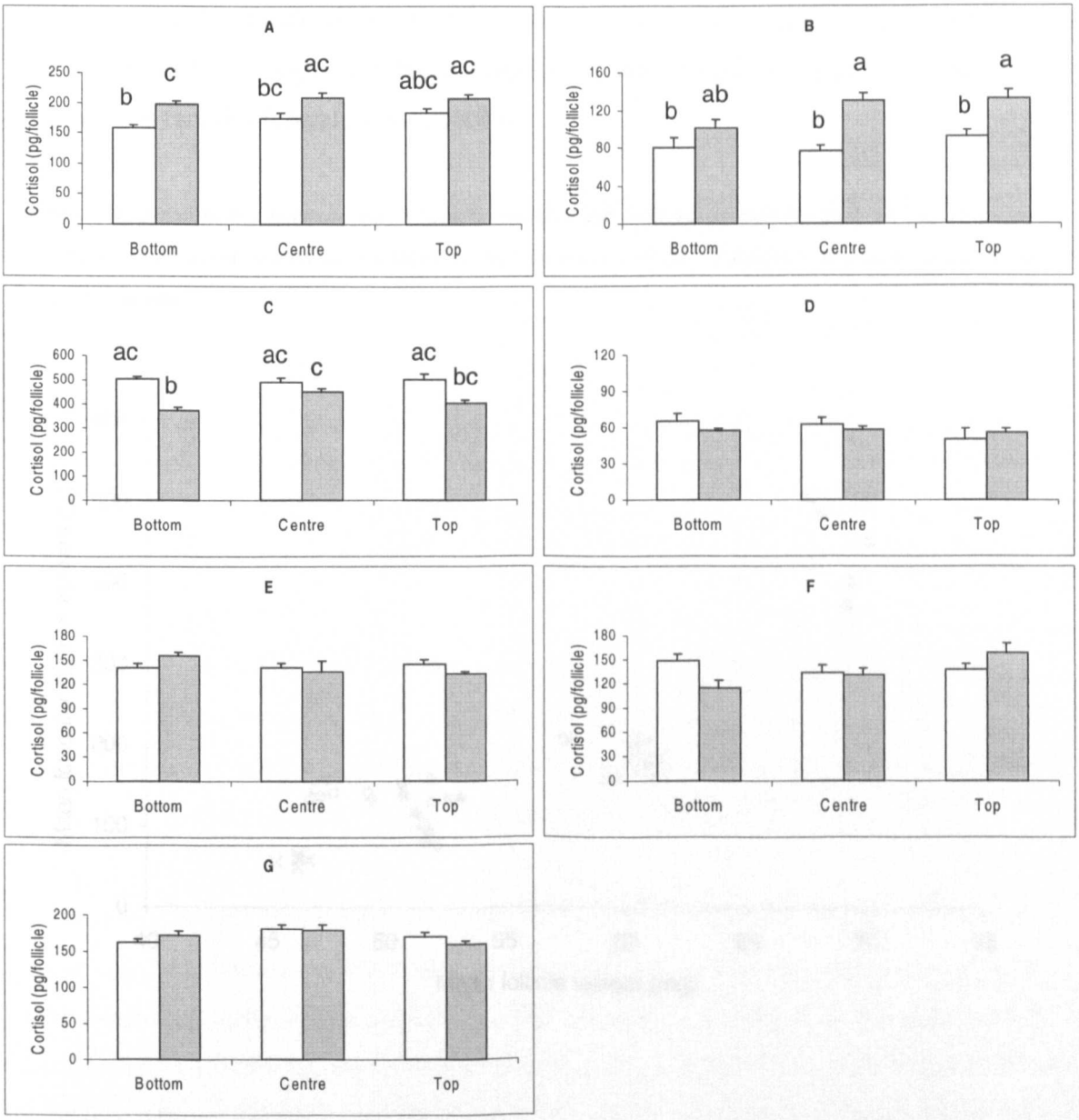


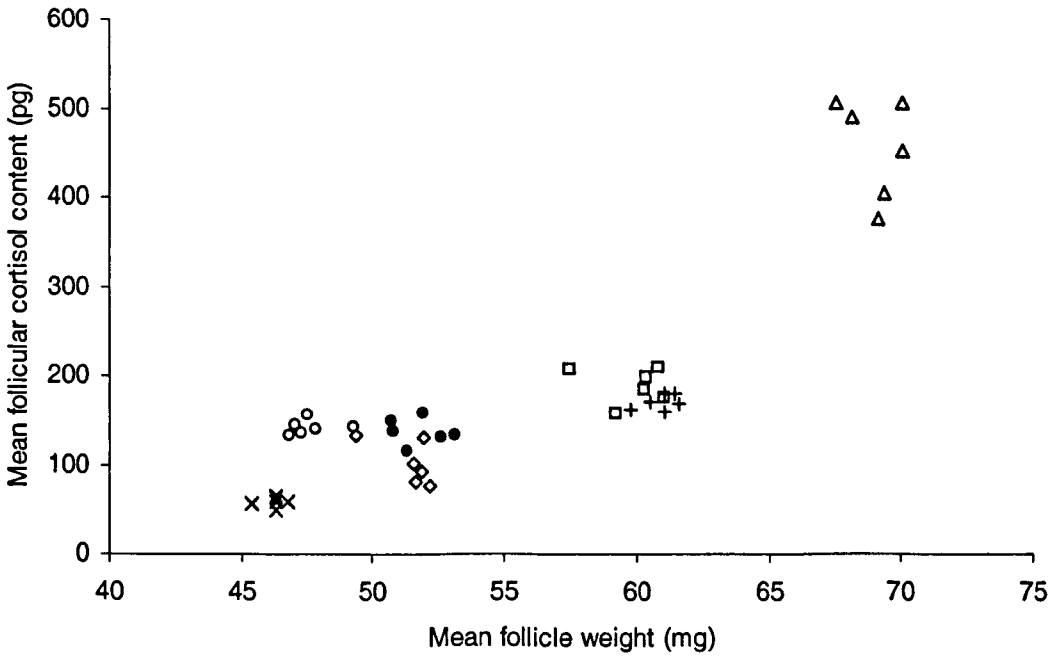
Figure 3.3. Regional variation in follicle cortisol content. Each graph represents follicles from a different female (maternity indicated by letter). Right ovary shaded. Columns represent the mean + SE. Columns marked with different letters are significantly different (One-way ANOVA and Tukey's post-hoc tests. $p<0.05$).



CVs of follicle weight and cortisol content were calculated for the six ovary regions in each fish, and these CVs used in separate 3-way ANOVAs to determine whether maternity, ovary, or ovary region influenced regional variability of follicle characteristics. Regional variability in follicle weight and cortisol content was

significantly different between females ($F_{6,32}$=9.70, p<0.001 & $F_{6,32}$=5.63, p<0.001, respectively), but ovary of origin (weight: $F_{1,32}$=2.82, p=0.103 & cortisol: $F_{1,32}$=1.88, p=0.179) and ovary region (weight: $F_{2,32}$=1.23, p=0.306 & cortisol: $F_{2,32}$=0.75, p=0.480) had no effect. Thus follicle variability differed between females, but was unaffected by longitudinal or ovary position within each female. There was no relationship between regional follicle weight CVs and cortisol content CVs within any of the seven females (correlations, p>0.05).

Figure 3.4. Relationship between mean follicle weights and cortisol content for each of the six ovary regions when all seven pre-ovulatory females are combined. Different symbols represent means from different females.



Ovulated females

Egg weight variability within the body cavities of ovulated females ranged from 3.45 - 7.02 %, while CVs for egg cortisol content ranged from 4.49 % to 26.30 % (Table 3.4.). These values were not significantly different from the CVs for follicle weight and cortisol content of pre-ovulatory females (2-tailed t-tests; p=0.89 and 0.96, respectively).

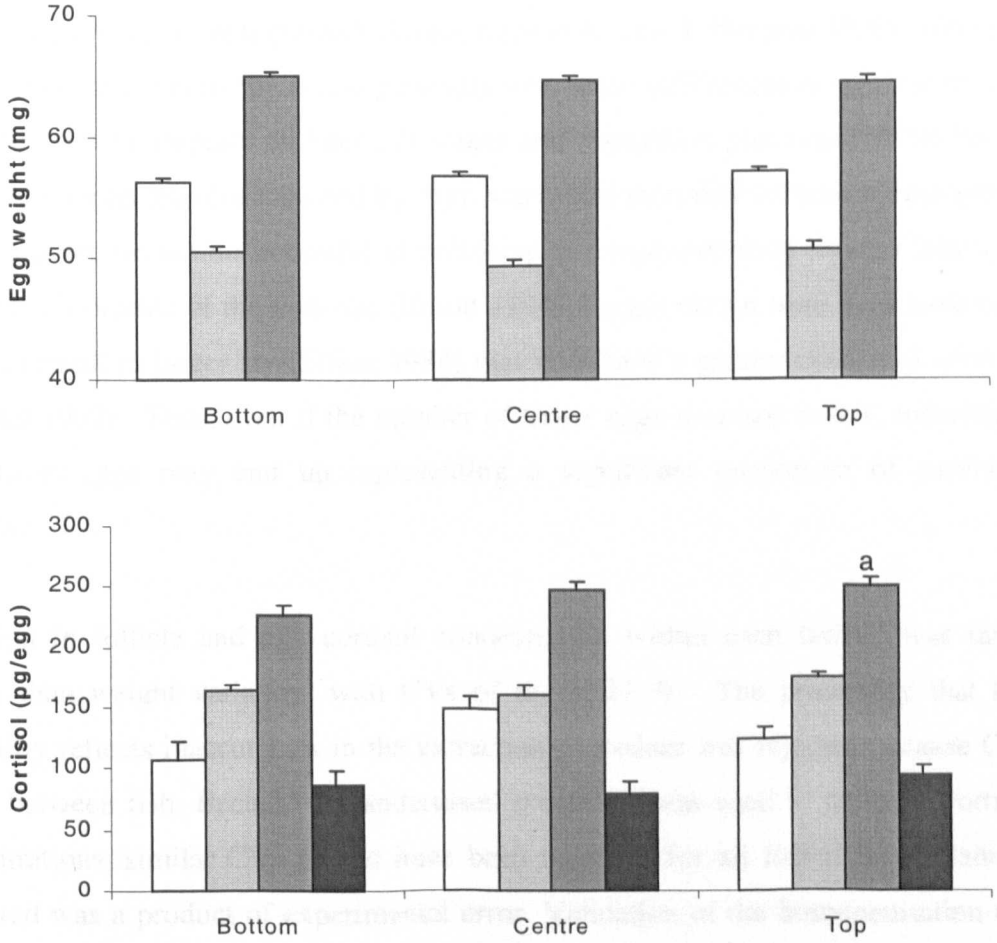
Table 3.4. Egg weight and cortisol (F) content data for the ovulated study animals. Mean values are given ± SE.

	Female			
	W	X	Y	Z
Mean egg weight (mg)	56.7 ± 0.2	50.2 ± 0.4	64.8 ± 0.2	-
CV (%)	3.58	7.02	3.45	-
Mean egg F content (pg)	127 ± 7	166 ± 4	241 ± 4	87 ± 5
CV (%)	24.80	9.44	4.49	26.30
Plasma F (ng ml^{-1})	3.43	3.82	3.27	-
Ovarian fluid F (ng ml^{-1})	1.34	-	1.66	2.85

Mean regional egg weights and cortisol content are illustrated in Figure 3.5. Although egg weight varied between ovulated females ($F_{2,279}$=710.92, p<0.001), there was no difference in the average weight of eggs from different regions of the body cavity ($F_{2,279}$=1.08, p=0.341). Egg cortisol concentrations varied between females ($F_{3,60}$=173.17, p<0.001), and also between body cavity regions ($F_{2,60}$=3.63, p=0.032). Interaction effects were not significant, indicating that the trend of regional differences in egg cortisol content was consistent between females. From Figure 3.5. it appears that cortisol content is higher in eggs from the anterior body cavity.

CVs for egg weight and cortisol content were calculated for each region of the body cavity for each female, and variation in these CVs in relation to region and female were examined using 2-way ANOVAs. The amount of variation in the data varied significantly between females (weight: $F_{2,8}$=37.04, p=0.003 & cortisol: $F_{3,11}$=11.07, p=0.007), but not between regions (weight: $F_{2,4}$=2.69, p=0.182 & cortisol: $F_{2,6}$=1.50, p=0.296).

Figure 3.5. Weight and cortisol content of eggs from different regions of the body cavity of four ovulated females. Each shade represents a different female (W→ Z from left to right). Columns represent the mean + SE. Region columns marked with different letters are significantly different from other regions of the same female (within-female one-way ANOVA and Tukey's post-hoc tests. $p<0.05$).



Discussion

Variation in egg size or egg cortisol content at spawning may contribute to individual variation in the physiology and behaviour of juvenile salmonids (e.g., Bagenal 1969, Thorpe *et al.* 1984, Einum & Fleming 1999, Chapter 2). The present study was intended to determine the extent to which natural variation in egg size and cortisol content occurs within a female, and thus whether there is the potential for these factors to contribute to individual variation in full-sibling juvenile salmonids. I assessed follicle variability within pre-ovulatory females and egg variability within ovulated females. While intra-female egg size variability has received attention in previous

studies (reviewed in Kamler 1992), this is the first investigation of intra-female variation in egg hormone concentrations.

I found that variation in follicle and egg weight within females was similar to that previously reported for the pre-fertilisation wet weight of eggs from wild brown trout (2.8-4.7 %, I.A. Fleming unpublished data, cited in Einum & Fleming 1999). Although intra-female variation in egg size is generally low, small differences in egg size may be sufficient to have impacts on later life stages and population processes. Wild brown trout populations are characterised by high starvation mortality following emergence, as many juveniles are unsuccessful at switching to exogenous food sources following complete absorption of the yolk-sac (Elliott 1994). Larger brown trout eggs have been found to result in larger fry (Elliott 1984) that may have a greater chance of survival (Bagenal 1969). Thus, even if the number of larger eggs in a nest is low, individuals from these eggs may end up representing a significant proportion of surviving progeny.

Variation in follicle and egg cortisol concentration within each female was much greater than weight variation, with CVs of up to 27 %. The possibility that this variability reflects inaccuracies in the extraction procedure was rejected because CVs varied between fish. Because a standardised procedure was used to measure cortisol concentrations, similar CVs should have been recorded for all fish if the variability measured was a product of experimental error. Validation of the homogenisation and solvent extraction steps of sample preparation showed that variation in the efficiency and accuracy of these procedures was low (Appendix 1). It is therefore evident that cortisol concentrations in eggs and follicles prior to spawning can vary within the ovaries and body cavities of females.

The range of CVs for egg and follicle weight, and for egg and follicular cortisol content, were the same. This suggests that egg weight does not become more uniform after loss of the follicle layer, and also that egg cortisol content does not become more uniform as eggs lie in ovarian fluid within the body cavity.

Female salmonids spawn multiple times over the course of several hours or days (Fleming 1998), and my observations suggest that the first spawning appears to

involve eggs at the rear of the body cavity only (Chapter 4). Thus, the distribution of variation in egg size or hormone content in relation to position within the body cavity or ovaries may have implications for inter- and intra-nest variation in egg characteristics. I therefore examined the weights and cortisol contents of follicles from the anterior (top), centre, and rear (bottom) of each ovary, to determine whether the distributions of eggs prior to spawning could influence the characteristics of eggs deposited into the first nest. Because only the left ovary extended into the posterior body cavity in the pre-ovulatory females examined in this study, I suggest that, if the females had been allowed to spawn naturally, eggs deposited into the first nest would originate from the bottom region of this ovary.

There were significant effects of ovary region on follicle weight and cortisol content, but these effects were not consistent between or within fish. Follicle weight and cortisol content did not consistently increase or decrease longitudinally along the ovaries, and the bottom region of the left ovary was not distinguished from other ovary regions by any consistent difference in follicle weight or cortisol content. There was a slight possibility that heavier follicles were located toward the centre of the ovaries, but examination of more females is required to determine whether this trend is real.

The relationship between position in the body cavity and egg weight and cortisol content was examined in a small number of ovulated females. Body cavity position did not appear to affect the weight of ovulated eggs, although the sample size of three females is inadequate to draw any general conclusions. Similarly, although cortisol content data was available from four females, cortisol content of eggs in the anterior region of the body cavity was higher than that in eggs from the centre and posterior regions of the body cavity. Assuming that ovulated eggs are tightly packed within the body cavity prior to spawning, and that the position of an egg within the body cavity prior to spawning reflects original ovary position, then finding higher cortisol concentrations in the anterior body cavity is not consistent with the results from pre-ovulatory females. This result in ovulated females may therefore be a misleading by-product of a small sample size, or could indicate changes in egg cortisol content during or following ovulation. If the eggs in the anterior body cavity really do contain more cortisol, then it is possible that eggs spawned into the first nest contain a lower amount of cortisol than eggs spawned in subsequent nests. Eggs from the anterior body cavity

of ovulated females are unlikely to be deposited together into the same nest (unless the female spawns only twice), as eggs are redistributed within the body cavity following the first spawning event (*pers. obs.*, Chapter 4). If the steroid content of eggs changes in the body cavity after ovulation, then steroid content could conceivably change between consecutive spawnings also, resulting in inter-nest variation in egg steroid content. This possibility will be examined in Chapter 4.

Ovary of origin had no significant effect on the weight of follicles, despite the right ovary of each pre-ovulatory female being smaller than the left. This relationship is very common (D.S. Keay, B. Campbell *pers. comm.*). Lateral displacement of the viscera within the body cavity may limit growth of the right ovary, reducing the number of oocytes developed, but not affecting their growth. The lack of any effect of ovary on follicle weight, despite significant regional variation in follicle weight being found in several females, raises questions about the mechanisms affecting oocyte growth in these fish. Vitellogenin (VTG) is synthesised in the liver and transported to the ovaries via the circulatory system (Wallace & Selman 1981, Wiegand 1996). At the ovaries, VTG is taken up by developing oocytes by receptor-mediated pinocytosis (Tyler & Sumpter 1996). Excess food was available to broodstock, and thus the resources required for vitellogenin (VTG) synthesis were likely to be unlimited. Mejen (1940, cited in Kamler 1992) has suggested that under such conditions, all oocytes situated in the vicinity of either large or small blood vessels will receive the same supply and quantity of nutrients required for growth, implying that oocyte size should be relatively uniform. As this was clearly not the case in these fish, I suggest that either I have quantified the degree of natural variation that occurs in a biological system despite optimal conditions, or that variation in proximity to large and small blood vessels may affect oocyte growth even under favourable trophic conditions.

The ovaries were highly vascularised, and the diameter of blood vessels appeared to vary greatly between different areas of the ovary. A large vessel, for example, traversed the dorsal ovary surface, while smaller capillaries were found within the ovary mass. With no reason to expect limited VTG synthesis in these fish, it is unlikely that oocyte growth in these fish was limited by the supply of VTG to the ovary. Instead, I suggest that oocyte growth in these fish was limited by the rate of VTG uptake by the oocytes (thus determining the maximum oocyte size) and the supply of

VTG to individual follicles. I hypothesise that oocytes in contact with larger blood vessels may be larger than oocytes from other parts of the ovary. Where follicles are closer to large blood vessels, VTG may be constantly replenished so that the factor limiting oocyte growth is the rate of VTG uptake. If the blood supply were more restricted, then VTG supply to the follicle, and thus uptake, could be reduced. This hypothesis would explain why there were differences in follicle weight between regions of an ovary, but not between ovaries. Proximity to blood vessels of different sizes could also affect follicular cortisol content, and explain the high intra-female variation in follicular cortisol content.

I would have liked to examine follicle characteristics in relation to the proximity of blood vessels of different size, but this was simply not practical. Individual follicles had to be pooled to provide sufficient tissue for hormone analyses, and these pools needed to be frozen as quickly as possible after the fish were killed. The time required to select follicles by their proximity to blood vessels would be truly prohibitive and, as proximity to blood vessels varies from follicle to follicle, difficulties would arise when trying to pool follicles for steroid analyses. Thus, whether local blood supply influences follicle weight remains an interesting area to be addressed.

The very different molecular natures of VTG and cortisol could explain the increased variation in follicular cortisol content relative to follicle weight. VTG is deposited in the developing oocyte over a period of months, and movement is unidirectional into the ovum. Cortisol, on the other hand, is likely to move in and out of the ova (Feist *et al.* 1990, and see Appendix 5). As levels of cortisol in the blood may fluctuate widely over relatively short time periods, this could create rapid temporal shifts in cortisol concentrations within the ovary. Variation in proximity to the blood supply could therefore result in variation of the exposure of follicles to fluctuations in plasma cortisol concentrations, and thus to high variation in the amount of cortisol contained in follicles within the ovaries of a single female.

Egg/follicle weight and cortisol content varied among females. Although there was a positive relationship between follicle weight and cortisol content across females, there was no consistent relationship between follicle weight and cortisol content within females. This result indicates a general relationship between follicle size and cortisol

content, but that the mechanisms responsible for oocyte growth and cortisol uptake are not tightly linked within a female. Theoretically, cortisol may enter the oocyte at any stage before spawning. Final oocyte size is largely determined by the amount of VTG taken up by the developing oocyte, and this uptake process has a distinct endpoint prior to ovulation (Tyler & Sumpter 1996). Thus cortisol uptake may continue beyond the point of VTG uptake, and cortisol content may change independently of oocyte size.

Although only cortisol concentrations were examined, other steroid hormones also pass into the developing oocyte. In mature females, testosterone is primarily produced in the follicle layers surrounding developing oocytes (Tyler & Sumpter 1996). Thus testosterone does not need to be transported into close proximity to the oocytes via the blood supply, although its production is dependent on blood-borne precursors and endocrine regulators of follicular steroidogenesis. The period of testosterone production is approximately the same as the period of VTG uptake. Therefore, during vitellogenesis, a positive relationship may exist between follicle weight and testosterone content within the ovaries of a single female. This relationship could weaken after the end of vitellogenesis if testosterone, like cortisol, can move both in and out of eggs.

Because I wanted to investigate the effect of developmental position within the ovaries on oocyte characteristics, I had to examine ovarian follicles. This introduces the question of whether the data reported here reflect differences between oocytes that would persist beyond ovulation, or whether these differences could be due to variation in the follicle layers. The follicles were post-vitellogenic (as evidenced by the position of the germinal vesicle), so the growth phase was over, and the only difference between the weight of these oocytes and their eventual egg weight is the weight of the follicle layer surrounding them. The follicle layer is very thin, and the ratio of spherical surface area to volume is such that, as oocytes increase in radius, the proportion of follicle weight contributed by the follicle layer decreases. From the pilot study that measured both follicle weight and diameter, it appeared that follicle diameter varied only slightly within a female, so regional differences in mean follicle weight were almost certainly due to differences in oocyte weight, and not to differences in the weight of the follicle layer. Regional differences in follicular cortisol

content could be attributed to differences in the cortisol content of the follicle layer rather than oocyte content. However, given that body cavity region affected egg cortisol content in ovulated females (where eggs have shed the follicle layer), and that egg and follicle cortisol concentrations were similar, I argue that the differences observed in intact females are related to oocyte content and not follicle layer content.

In summary, this study has demonstrated that there is intra-female variation in the weight of eggs and follicles of hatchery-reared mature brown trout. Follicle weight differed between females, and was affected by position within the ovary, although positional effects were not consistent between females. There was a greater degree of intra-female variability in egg and follicle cortisol concentrations than in egg and follicle weight. The amount of cortisol varied among females (there was a positive relationship between follicle weight and cortisol content), and between ovary and body cavity regions within females. In pre-ovulatory females, this regional variation is not consistent, whereas eggs from the anterior region of the body cavity of ovulated females appear to have relatively high cortisol contents. The latter results must be viewed with caution given the small sample size. Cortisol content and weight did not appear to be closely related within females. So, if egg size and cortisol content can influence juvenile physiology and behaviour, then the observed intra-female variation in weight and cortisol content of eggs/follicles provides a possible explanation for individual variation in the physiology and behaviour of full-sibling brown trout. The absence of a clear relationship to developmental position along the longitudinal axis of the ovary suggests that variation in egg characteristics within females contribute much more to within- than between-nest variation in naturally spawning females.

CHAPTER FOUR

DOES SPAWNING ORDER MATTER?
I. INTER-NEST DIFFERENCES IN THE STEROID CONTENT, SIZE, AND PRE-HATCHING VIABILITY OF BROWN TROUT EGGS

Abstract

Variation in brown trout egg size and steroid content may contribute to variation in the physiology and behaviour of juveniles. In the wild, female salmonids will generally spawn multiple times over the course of several hours or days. Here I investigated whether egg size and hormone levels varied between nests spawned by pairs of wild brown trout in an artificial stream, and whether there were nest-based differences in the pre-hatching viability of offspring. Eggs were collected from 27 nests spawned by 7 pairs. Pairs spawned 3-5 times, generally at 8-12 hour intervals. There were no significant differences in egg size between nests of any of the pairs of fish at spawning, but egg cortisol, testosterone and 17α,20β-dihydroxy-4-pregnen-3-one content varied significantly but inconsistently among nests of some pairs. Under hatchery rearing conditions, eggs spawned last were less viable than eggs spawned first in 5 of 7 families. The potential therefore exists for full-sibling progeny spawned into different nests to display differences in early viability and hormone-induced differences in physiology and behaviour. Thus, spawning order may shape the development of individual variation in juvenile success and life history strategies.

Introduction

As discussed previously, individual juvenile salmon and trout can vary greatly in ecologically important aspects of their physiology and behaviour. In Chapter 3, I demonstrated considerable intra-female variation in brown trout egg steroid levels prior to spawning. If these differences persist at spawning, or others develop, then intra-female variation in egg hormone content could generate individual variation in the physiology and behaviour of full-siblings.

Variation in the steroid content of eggs from a single female might arise during the spawning period, following ovulation of the eggs and their release into the body cavity, but prior to oviposition. Between ovulation and the end of spawning activity, plasma steroid concentrations of female rainbow trout (*Oncorhynchus mykiss*) go through rapid and extreme changes (Liley *et al.* 1986b). Plasma levels of testosterone and estradiol-17β, high shortly before spawning, were found to decline in ovulated and sexually active females, reaching lowest levels in postspawning fish. Concentrations of

17α,20β-dihydroxy-4-pregnen-3-one (17,20bP), a progestogen involved in final oocyte maturation in salmonids (Goetz *et al.* 1987), rose markedly in ovulated females allowed to dig nests and interact with sexually active males.

Can these changes in plasma steroid levels be communicated to the eggs? Once ovulated, eggs lie within the body cavity, surrounded by ovarian fluid. Feist *et al.* (1990) reported that concentrations of sex steroids in ovulated eggs reflected concentrations in the ovarian fluid. I have demonstrated that egg cortisol content increases in response to experimental elevation of ovarian fluid cortisol concentration, and that cortisol may be able to pass out of the eggs when transferred to unmanipulated ovarian fluid (Appendix 5). If steroids can move in and out of ova along a concentration gradient, then egg steroid concentrations might fluctuate in concert with changes in the steroid composition of the ovarian fluid. Thus, if changes in plasma steroid concentrations can affect concentrations of steroids in ovarian fluid, a mechanism exists whereby the steroid content of ovulated eggs could change prior to spawning. Another possibility is that eggs, once released into the ovarian fluid, might slowly take up or lose steroid across the egg membrane, with amount of uptake/loss dependent on the time spent in the ovarian fluid before spawning.

Many female salmonids display nesting behaviours during spawning in the wild (Elliott 1994, Fleming 1998). The female digs a shallow nest in gravel substrata using the caudal fin and sideways flexions of the body, before depositing a proportion of her eggs (Liley *et al.* 1986a). These are fertilised by the attending male, and the female then moves upstream and begins to cover the nest. In the process of so doing, she begins to excavate a second nest depression. In this way, females may spawn several times over a period of hours or days, creating a series of nests in an upstream direction (Fleming 1998). If egg steroid concentrations change during the course of spawning, then egg steroid levels could vary between nests. If this is the case, and the concentration of those steroid hormones is important in development, then there could be hormone-induced variation in the development of sibling offspring from different nests.

Disregarding any potential hormone differences, several studies on salmonids have shown that if eggs are retained within the body cavity for too long after ovulation, they become 'overripe' and less viable (Escaffre *et al.* 1977, Springate *et al.* 1984, de Gaudemar & Beall 1998, Lahnsteiner *et al.* 2000). Thus eggs spawned soon after ovulation may be more viable than eggs spawned last. This study examines the degree of nest-related variation in egg hormone levels, weight, and pre-hatching viability.

Methods

Broodstock

Wild adult non-migratory brown trout were captured from a hill loch near Pitlochry, Perthshire, Scotland during November 2000, and transferred to the Fisheries Research Services Freshwater Laboratory unit at Almondbank, Perthshire, Scotland. Size-matched pairs of 1 male and 1 female (n=7) were anaesthetised with benzocaine (25 $mg.l^{-1}$), weight and length measured, and transferred to spawning channels (see below). The females were assessed as still having intact ovaries, but being within 2 days of ovulation, by the tautness of the abdomen and appearance of the anal vent. The males used in the study expressed milt when gentle pressure was applied to the abdomen. Using only one pair in each channel section eliminated intrasexual competition for spawning opportunities and ensured that all progeny from a single female would be full-siblings (see Chapters 5 & 6). Details of fish size and spawning activity are presented in Appendix 7.

Spawning channels

This experiment took place in sections of a flow-through, artificial stream channel fed by the Almond River (Perthshire, Scotland). The entire channel is 80 m long by 1.5 m wide, with viewing windows along one side. It is possible to control abiotic factors such as light, flow rate, water height, and substratum type and size. The channel was divided longitudinally with a solid wooden partition, and steel mesh screens were inserted between the side window and longitudinal partition to create identical adjoining experimental sections (each 3.6 m long x 0.6 m wide). The floor of each section was covered with black polythene. Gravel (2-8 cm diameter, 10 cm deep) lined the anterior 3.3 m of each section. In the last 0.3 m of each section, solid concrete blocks (thickness 15 cm) were placed in the stream and covered with more polythene

to prevent fish spawning against the back mesh. The water was maintained at a depth of 25 cm and at a flow rate of 25 $cm.s^{-1}$. The photoperiod roughly mimicked the ambient light regime for November at this location, channels being in darkness between 1730 and 0800 h. The sections were covered with soft mesh screens to prevent the fish escaping, and a plywood board (90 x 60 cm) was laid across the width of each channel to create overhead shelter and shadow for the fish.

Spawning behaviours

Spawning behaviours (Tautz and Groot 1975, Liley *et al.* 1986a), fish position and female abdominal appearance were recorded at least once every two hours following introduction of fish into the spawning channels, to determine the location and timing of spawning events. To allow observation of nocturnal activity, a narrow-beam torch fitted with a red filter was used to scan the sections. The fish did not appear to react (by aversive movements or 'freezing') to this beam of light.

Nest excavation

Four to six hours after each spawning, a worker standing on the non-gravelled side of the longitudinal division excavated the eggs. Nets were placed across the channel upstream and downstream of the nest to capture any dislodged eggs and prevent movement of fish into the nest area during excavation. The gravel covering and surrounding the nest was gently scooped into buckets and transferred to a wet lab where it could be sieved (mesh size 1 cm) over a water-filled plastic container. To ensure that all eggs had been removed, gravel surrounding the nest was gently stirred up by hand so any loose eggs passed into the downstream net. Care was taken to minimise disturbance to any nests under construction, and gravel was replaced to resemble the appearance of the redd before excavation. No nests under construction were abandoned following excavation interruptions. Eggs from each nest excavated were kept separate at all times.

All nests were excavated at least 4 hours post-spawning to ensure that measures and handling were as consistent as possible between nests. Depending on how soon after an observation the fish spawned, the eggs could be excavated up to 6 hours post-spawning. A pilot study showed that eggs became fully hydrated and water hardened within 4 hours of spawning, and that there was little change in egg weight or cortisol

content between 4 and 24 hours post-spawning (Appendix 6). Water hardening is the process whereby eggs rapidly absorb water across the egg membrane, causing the egg to increase in size and the outer membrane to become stronger or 'hard'. If eggs were excavated too soon after oviposition, they would not be fully water hardened and weight measurements from different nests might give misleading results. Also, by standardising the time to excavation, clearance of hormones from eggs should be similar for all nests.

When no new digging activity had been observed for 48 hours following the last spawning event, the fish were netted and removed from the channel. Females were anaesthetised as above and blood-sampled (from the caudal vein using a needle and syringe) within 5 minutes of removal from the spawning channel. Length and weight of males and females were measured. The female was stripped of any remaining ova, and ovarian fluid samples were taken for hormone analyses. The fish were returned to a holding tank to recover from spawning.

Egg processing and husbandry

Eggs from each nest were processed as follows. The total number of eggs was counted. Most of the eggs were transferred directly to a container in a flow-through trough for rearing. Water from the Almond River was supplied to the troughs at ambient temperature at a rate of 2 $l.min^{-1}$. A sub-sample of 20-50 eggs was blotted dry, the eggs weighed individually and pooled in Eppendorf tubes to give samples with a total weight of approximately 0.2 g (2-5 eggs depending on egg size). These pooled samples were frozen at –20 °C for hormone analyses. Fertilised eggs were treated with extreme care at all times, and movement of ova was completed within 0.5 hours of excavation.

The eggs were checked regularly during development in the hatchery, with inviable eggs (milky-opaque) being removed, and the time taken to reach different developmental and life stages (e.g., eyeing, hatching) noted. When the eye-spots were well pigmented, 10 eggs from each nest were transferred to the University Field Station at Rowardennan, East Loch Lomondside (see Chapter 6), while the remainder were reared on at Almondbank until just before first-feeding (see Chapter 5).

Steroid extraction and quantification

Each egg sample was thawed at room temperature, and the egg membranes disrupted by hand using 20 strikes of a mounted needle (see Appendix 1 for further details). Sodium hydroxide (200 µl of 0.1M) was then added to each Eppendorf tube, and the samples vortex mixed for 30 s. After 1 h at room temperature, 1.0 ml of ethyl acetate was added to each Eppendorf tube. The tubes were capped and vortex mixed for 30 s, then centrifuged at 13000 rpm for 5 min to separate the phases. 0.9 ml of the upper, solvent, phase was transferred into a clean, labelled Eppendorf tube, and a further 0.5 ml of ethyl acetate was added to the sample Eppendorf tube. After vortex mixing, the sample was again centrifuged, and 0.5 ml of solvent was removed into the same extract Eppendorf tube. Eppendorf tubes were capped between extractions to prevent solvent evaporation. Plasma and ovarian fluid steroids were extracted by transferring a known volume of plasma or fluid to a clean Eppendorf tube and adding solvent as described above. Validation of the homogenisation and extraction procedures used are given in Appendix 1.

Concentrations of cortisol, testosterone, and 17α,20β-dihydroxy-4-pregnen-3-one (17,20bP) in the extracts were measured using radioimmunoassay, following the method described in Appendix 2. Serial dilutions of egg extracts showed that the dilution curves were not parallel to the standard curve at higher extract volumes. Egg testosterone concentrations in most families (defining a 'family' as all eggs produced by one breeding pair) were below the level of detection for the assay if extract volumes on the parallel part of the curve were used. I therefore measured testosterone in the same volume of extract for each sample, and made the assumption that materials interfering with the radioimmunoassay were present in equal quantities in all samples from a single female. Thus within-female comparisons should be valid, although reported values of testosterone may be erroneous. Between-family comparisons of testosterone concentrations should be regarded with some caution, as the degree of deviation from parallelism may vary between egg extracts from different females. Concentrations were not corrected for extraction efficiencies (Appendix 1).

Ethical note

The experiment was carried out under a U.K. Home Office Licence (PPL 60/2025). During spawning, fish were observed every two hours. Fish could have been removed if any kind of physical damage or aggression had been observed, but this was never necessary. Fish are not protected by the U.K. animal welfare legislation until they commence exogenous feeding, but all efforts were made to ensure that eggs and larvae were reared under optimal growth and welfare conditions.

Results

Broodstock, spawning and maternal steroids

Each pair of wild fish spawned 3-5 times, with an interval of at least 8 hours between spawnings, and a mean interval of 724 ± 104 minutes (12 h 4 min ± 1 h 44 min; see Appendix 7 for full details). By stripping the females after the end of spawning activity, it was clear that all females had spawned to completion, with less than 5 eggs remaining in the body cavity. The number of eggs spawned by each female generally declined between consecutive nests (Table 4.1.). Eggs deposited during each spawning were clearly those that had been lying in the posterior body cavity, as evidenced by the hollow appearance of this region immediately after each spawning. It appeared that subsequent covering and digging activities redistributed the eggs within the body cavity prior to the next spawning. The entire abdomen appeared flaccid after the final spawning.

Table 4.1. Number of eggs spawned by each pair of brown trout. (d) or (n) indicates whether the eggs were spawned during day (0800-1730) or night, respectively.

Pair	Nest 1	Nest 2	Nest 3	Nest 4	Nest 5	Total
1	226 (d)	59 (n)	10 (d)	-	-	295
2	324 (d)	78 (n)	35 (d)	-	-	437
3	204 (d)	147 (n)	69 (d)	-	-	420
4	224 (n)	131 (d)	49 (n)	26 (n)	-	430
5	247 (n)	232 (d)	122 (d)	64 (n)	-	665
6	162 (n)	162 (d)	138 (n)	85 (n)	37 (d)	584
7	163 (n)	148 (d)	48 (n)	78 (n)	46 (n)	483

Blood and ovarian fluid samples were collected from each female 48 h after spawning activity had ceased. There was considerable variation among females in the concentrations of cortisol, testosterone and 17,20bP in plasma and ovarian fluid (Table 4.2.). There were no relationships between female weight and plasma or ovarian steroid concentrations (correlations, $p>0.05$). Female plasma and ovarian fluid cortisol concentrations were positively correlated ($r=0.842$, $p<0.05$ with 4 d.f.), but there were no associations between concentrations of 17,20bP in plasma and fluid. Associations between testosterone concentrations could not be examined because of the low levels present in plasma and fluid.

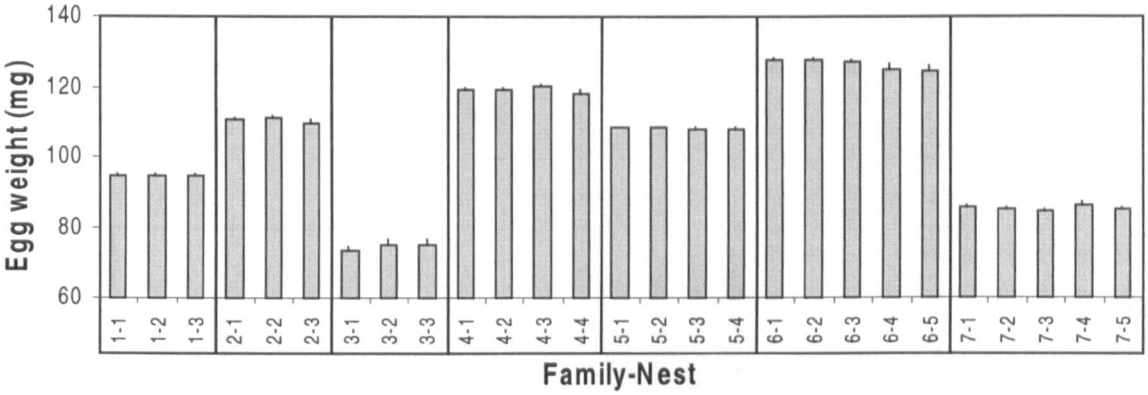
Table 4.2. Maternal plasma and ovarian fluid steroid concentrations (ng/ml) at the end of spawning. F – cortisol, T- testosterone, 17,20bP – 17α,20β-dihydroxy-4-pregnen-3-one. ND – non-detectable (<0.03 $ng.ml^{-1}$). Ovarian fluid was not collected from the female of pair 1.

	Plasma			Ovarian Fluid		
Pair	F	T	17,20bP	F	T	17,20bP
1	13.98	0.41	8.07	-	-	-
2	14.30	ND	9.85	9.38	2.25	0.66
3	17.82	4.03	32.44	4.81	4.01	1.36
4	18.46	ND	5.66	9.59	ND	0.91
5	22.83	ND	4.16	17.73	ND	3.67
6	12.04	0.93	38.58	3.47	ND	2.48
7	13.06	ND	6.70	1.89	ND	3.19

Egg weight variation

One-way ANOVAs were used to assess potential within-pair, inter-nest differences in mean egg weight. There were no significant differences among nests within any of the seven families (Figure 4.1.).

Figure 4.1. Mean (+ SE) weight of eggs recovered from each nest spawned by seven pairs of brown trout.



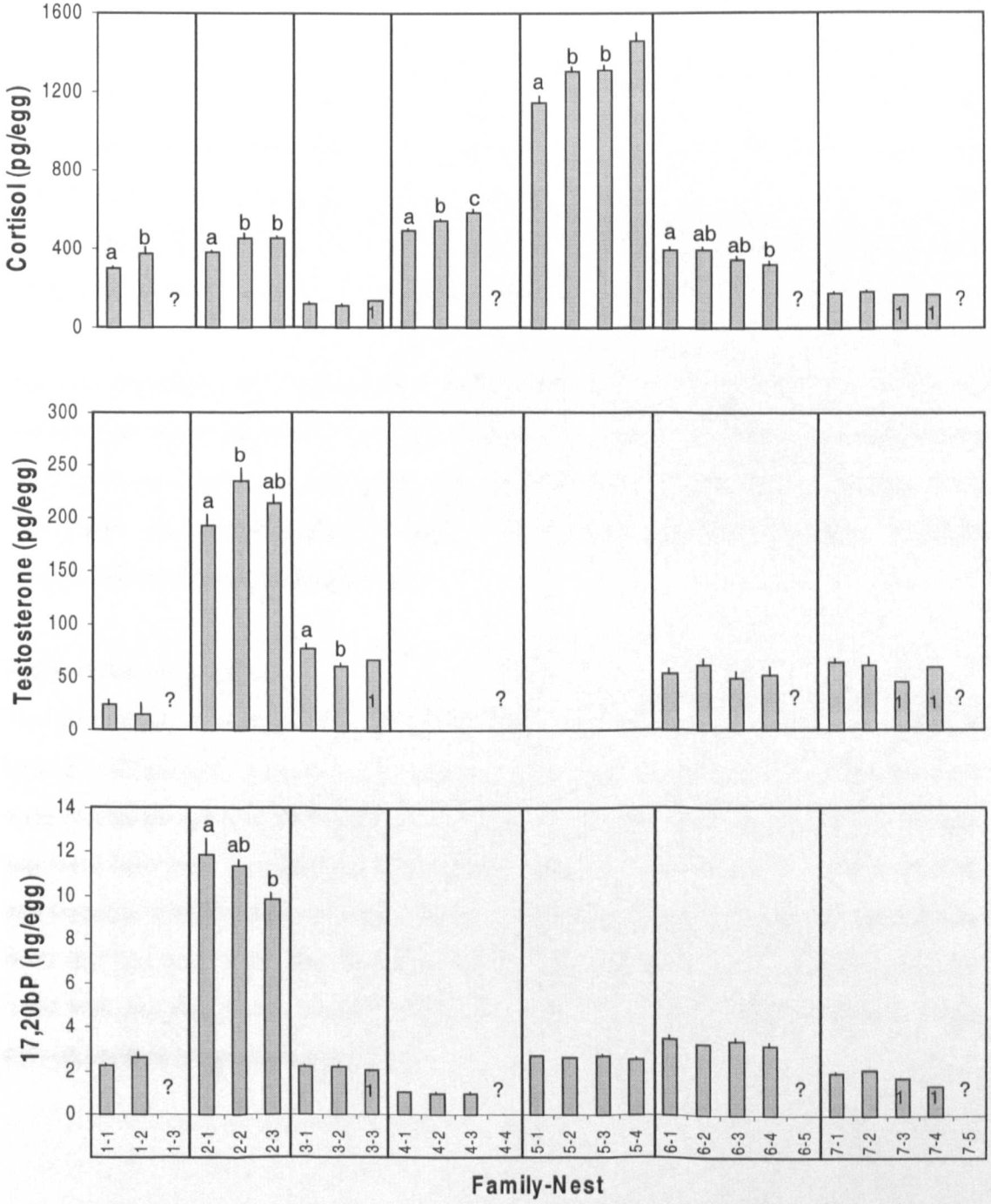
Egg steroid content

Egg steroid content varied between eggs of different females (Figure 4.2.). There was a positive association between egg and ovarian fluid cortisol concentrations (r=0.858, p<0.05 with 4 d.f., egg cortisol content corrected for inter-family differences in egg size). Mean testosterone or 17,20bP content of eggs from each female were not related to post-spawning maternal plasma and ovarian fluid steroid concentrations. Across families, there were no associations between mean concentrations of the different steroids within the eggs (Spearman's rank correlations, p>0.05). Egg 17,20bP concentrations were much higher than cortisol or testosterone concentrations (note different y-axis units in Figure 4.2.).

Within the eggs of each family, cortisol and testosterone, and testosterone and 17,20bP concentrations were unrelated (ANCOVAs with nest number as a factor, p>0.05). However, in eggs from family 6, 17,20bP concentration positively covaried with cortisol concentration ($F_{1,15}$=6.35, p=0.024; concentrations expressed as steroid per gram egg to correct for any differences in egg weight).

One-way ANOVAs with *post hoc* Tukey's Tests were used to assess data from each pair for differences in egg steroid content between nests. Mean egg steroid contents of each nest measured are illustrated in Figure 4.2. Having offspring for use in Chapters 5 and 6 was the experimental priority, so where egg numbers were low, egg steroid content was not assessed.

Figure 4.2. Mean (+ SE) steroid content of eggs recovered from each nest spawned by seven pairs of brown trout. 17,20bP - 17α,20β-dihydroxy-4-pregnen-3-one. Note different units for 17,20bP. '?' indicates insufficient eggs to allow steroid analyses. Missing bars represent values that were below the limits of detection. Within nests from a single family, columns marked with different letters are significantly different (One-way ANOVA and Tukey's tests with a family error rate of 0.05). Except where indicated (by numbers in columns), n=6.



There were significant inter-nest differences in egg cortisol content in eggs from five of the seven families. The trend of inter-nest differences was not consistent between families, although there did appear to be a constant increase or decline in egg cortisol content between the first and last nests measured for each of these five pairs. Eggs spawned in the first nest of four families contained significantly less cortisol than eggs spawned in later nests.

Significant inter-nest variation in testosterone content was found in eggs from two pairs of fish. In one family, eggs spawned in the first nest had lower testosterone concentrations than eggs spawned in the second nest while, in the other family, egg testosterone content was higher in eggs spawned first than in those spawned in the second nest. Testosterone content was so low as to be non-detectable in eggs from another two pairs.

Egg concentrations of the progesterone derivative 17,20bP appeared to be generally unrelated to spawning order. However, significant inter-nest variation was detected in eggs from one pair. In this case, egg 17,20bP content appeared to decline with subsequent spawnings, and eggs spawned first had significantly higher 17,20bP concentrations than eggs spawned last.

Pre-hatching egg viability

The percentage of viable offspring at hatching was calculated for each nest (100 x # hatched offspring/# Almondbank reared eggs), and these percentages (Figure 4.3.) were examined for an effect of spawning order. In general, viability was lower in eggs spawned later than in eggs spawned earlier. However, a paired t-test (a=0.05) of first and last nest viability rates for each family (excluding family 1 because all individuals from the last nest were transferred to the University Field Station) showed that this trend was not significant (t=2.19, p=0.080, n=6). Egg viability was unrelated to egg steroid content (regressions, p>0.05).

Figure 4.3. The percentage of offspring from each nest spawned by seven pairs of brown trout that were viable at hatching. Sample sizes are given in Table 4.3.

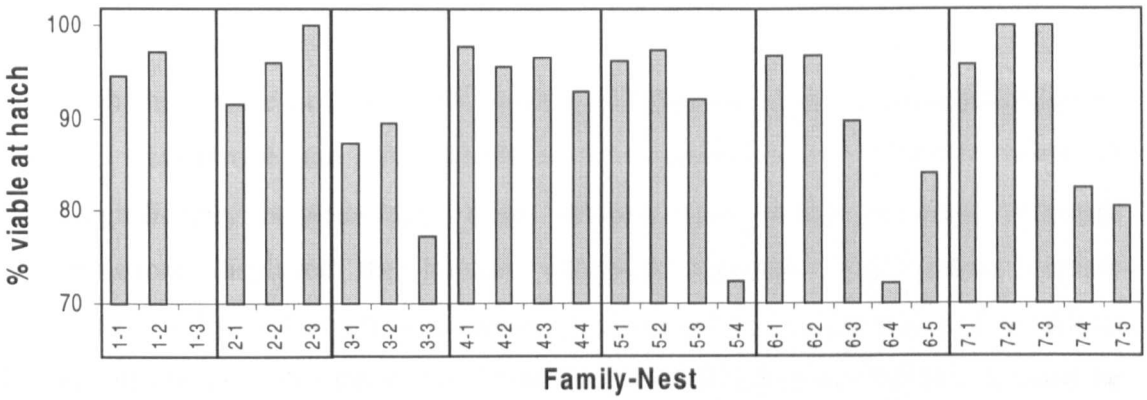


Table 4.3. Number of offspring from each nest reared entirely at Almondbank.

Pair	Nest 1	Nest 2	Nest 3	Nest 4	Nest 5
1	167	35	0	-	-
2	261	50	10	-	-
3	150	96	44	-	-
4	184	91	29	14	-
5	213	192	102	40	-
6	122	122	98	65	25
7	115	106	48	57	46

Discussion

There is marked individual variation in juvenile salmonid physiology and behaviour both between and within full-sibling families (Metcalfe *et al.* 1995, Cutts *et al.* 1998, Metcalfe 1998). This individual variation may be due to variation in concentrations of maternal steroids in the eggs at fertilisation. I have shown that the steroid content of eggs from different females can vary considerably (Chapters 2 & 3, and the present chapter also). In Chapter 3, I demonstrated that variation in egg cortisol content also occurs within the intact ovaries of mature brown trout. If these differences are maintained until spawning, then a potential mechanism exists for the generation of

hormone-induced variation among full-siblings. This chapter reports that there can be post-spawning variation in the steroid content of eggs from single females. Thus, there is the potential for hormone-induced variation among full-sibling progeny.

Although there were no consistent patterns of hormone variation, concentrations of cortisol, testosterone and 17α,20β-dihydroxy-4-pregnen-3-one (17,20bP) could all vary significantly between eggs from different nests within families. Cortisol concentrations displayed the greatest inter-nest variation. Egg cortisol content increased in a linear fashion as spawning progressed in four families, and decreased linearly in another. No trend was found in the remaining two families. It must be remembered that steroid measures were not available for the last nest of four families, however. In the two families in which significant inter-nest variation in testosterone was found, there was no consistent trend. Significant inter-nest variation in egg 17,20bP content was found in one family, with concentrations in eggs spawned first being higher than those in eggs spawned last. There was no relationship between the cortisol and testosterone content of eggs, but concentrations of egg cortisol and 17,20bP concentrations did covary in a positive fashion in eggs from one family, suggesting that the factors responsible for inter-nest variation in egg cortisol content in this family may also have been responsible for inter-nest variation in egg 17,20bP concentrations.

Is the inter-nest variation observed in this experiment related to pre-ovulatory variation in egg steroid concentrations, or did the variation arise after ovulation and between spawnings? Although Chapter 3 identified regional differences in follicular cortisol content within intact females, these differences were not consistent between females. It therefore seems unlikely that the common trends in inter-nest variation in egg cortisol content observed in this experiment could be due to pre-ovulatory egg distributions. I have demonstrated that Atlantic salmon (*Salmo salar*) egg cortisol content can change in response to changes in ovarian fluid cortisol concentrations, with cortisol apparently able to move both in and out of ovulated eggs (Appendix 5). I therefore suggest that the changes in egg steroid content observed in this experiment occurred between spawnings. If so, these changes may be in response to changes in maternal plasma and ovarian steroid concentrations, could perhaps be a consequence of changing fluid:egg

ratios within the body cavity, or may be related to the length of time eggs are retained prior to spawning. Without sampling ovarian fluid and plasma steroid levels during the course of spawning, this argument remains entirely speculative.

What factors might affect steroid concentrations in maternal plasma and ovarian fluid? Plasma cortisol concentrations are normally high in mature female salmonids during the final stages of ovarian development (Leatherland 1999), but exposure to external stressors may have the effect of further elevating cortisol levels (Campbell *et al.* 1994, Stratholt *et al.* 1997). The process of nest excavation may represent such a stressor, in which case the steady increase in egg cortisol content from first to last-spawned nests could be explained. Alternatively, if movement of steroids from ovarian fluid to eggs is a slow process, then increased cortisol content in eggs spawned later could simply reflect the time spent in ovarian fluid prior to spawning. Given that egg cortisol content decreased between spawnings in one pair, the answer is obviously not a simple one.

Liley *et al.* (1986b) described the changes in rainbow trout maternal sex steroids during the spawning period. Plasma concentrations of androgens (such as testosterone) decline prior to ovulation and are at low levels during the spawning period. If ovarian fluid steroid concentrations reflect plasma steroid levels, then testosterone concentrations at ovulation may be higher in the eggs than in the surrounding ovarian fluid. If testosterone is able to move out of the eggs in response to such a concentration difference, then I might predict a decrease in egg testosterone content between nests. No such trend was consistently found, however, and in one case, the opposite was true – egg testosterone concentrations increased after the first spawning. Why might this be? Testosterone is produced by cells in the follicle layer, with synthesis reaching a peak prior to ovulation (Scott *et al.* 1980, 1983, Fostier & Jalabert 1982, Scott & Baynes 1982). It is unlikely that post-ovulatory follicles perform a last burst of testosterone synthesis during spawning, as biosynthetic pathways have switched to the production of 17,20bP (Fostier *et al.* 1983). I hypothesise that inter-nest differences in egg testosterone content are the result of complex diffusion processes between plasma, ovarian fluid and eggs. A study where samples are taken at a series of time points over the course of spawning activity would allow proper investigation of this hypotheses. This conclusion is supported by the

presence of large amounts of testosterone in the ovarian fluid (and eggs) of one female, but not in the plasma.

Concentrations of circulating 17,20bP are low prior to final maturation and begin to increase just before the eggs resume meiosis. Levels increase dramatically during this phase, and may continue to increase during ovulation (Goetz *et al.* 1987). In some *Onchorhynchus* species there are indications that post-ovulatory follicles may retain the capacity to synthesise 17,20bP for several days following ovulation (Young *et al.* 1983). Liley *et al.* (1986b) and Liley & Rouger (1990) demonstrated that 17,20bP is associated with the synchronisation of maturational and behavioural events surrounding spawning in rainbow trout. Plasma 17,20bP levels increased in newly-ovulated females when mature males and spawning substrata were made available. Plasma 17,20bP remained high until most of the eggs were spawned, decreasing to low levels on completion of spawning.

During this experiment, all pairs completed spawning within 2-4 days, within the period when post-ovulatory follicles may still have been producing 17,20bP. However, as I did not collect plasma or ovarian fluid samples during spawning (to avoid disturbance to the fish as much as possible), I was unable to assess changes in maternal steroid levels. In all but one family, there were no significant differences in the 17,20bP content of eggs from different nests. This may indicate either that 17,20bP levels remained constant throughout spawning, or that 17,20bP cannot move as easily as cortisol across ovulated egg membranes. In the one family in which there were significant differences in the 17,20bP content of eggs from different nests, 17,20bP levels decreased between subsequent spawnings. This result can be explained if 17,20bP passed out of the eggs in response to a decline in ovarian fluid concentrations. As mentioned above, egg concentrations of cortisol and 17,20bP covaried in one family, suggesting that the factor(s) affecting movement of different steroids may be the same in this family.

In addition to inter-nest variation in egg steroid content, spawning order may also affect egg viability. Although not significant, there were indications that eggs spawned first by a female could be more viable than eggs spawned last. Several previous studies have indicated that the length of time ovulated eggs are retained

within the abdominal cavity can negatively affect viability (Escaffre *et al.* 1977, Springate *et al.* 1984, de Gaudemar & Beall 1998), but these studies have only found effects over much longer periods (10-20 days, generally). The current study, however, is the first to directly investigate egg viability in relation to spawning order. Within 24 hours of fertilisation, it is relatively safe to move eggs as long as they are treated gently. After this time, mechanical shock can cause embryo death (Jensen & Alderdice 1983, Laird & Needham 1988). All eggs in our study were moved within 6.5 h of spawning, and were treated with the utmost care, so mechanical shock is unlikely to be a factor affecting our viability data. However, the viability data could be affected by milt quality and male behaviour. It is possible that male attentions to the female and fertilisation success could vary between spawnings. Because eggs were termed inviable if they turned milky-opaque or failed to hatch, infertile eggs were not always distinguishable from dead ones. But is the potential variation in egg viability reported here actually ecologically relevant?

Viability of eggs from all nests was greater than 70 %, and none of the fish held at Almondbank died following hatching. In Chapter 2, brown trout reared under identical conditions until the September of their first year also had negligible mortality rates. How does this compare with a natural situation? Elliott (1987) presents survival data from a long-term study of brown trout in Wilfin Beck. Data suggest that, by August/September of their first year, only 3.9 % of the eggs originally spawned survived. Thus, the (predominantly) slight variations in nest-related egg viability reported here probably have very little relevance under natural conditions. Indeed, under natural conditions, differences in nest quality are far more likely to generate variation in the survival of sibling offspring than subtle differences in egg quality. Factors such as nest depth, interstitial water flow, and covering depth can affect egg survival (see reviews by Milner *et al.* 1981, Crisp 1989). By retrieving the eggs and rearing them under hatchery conditions, I examined intrinsic differences in egg quality at the expense of examining variation in offspring survival within the spawning channel.

In Chapter 2, it was not clear whether artificial elevation in egg testosterone or cortisol content could influence juvenile metabolic rate, size and social status. The control levels of cortisol and testosterone of eggs in Chapter 2 and egg steroid concentrations

in this chapter were comparable. Inter-nest differences in egg testosterone content reported here (*c.* 10-45 pg) were less than the experimental elevations induced in Chapter 2 (100-450 pg). If the effects observed in Chapter 2 were treatment-related rather than tank effects, the question of whether the differences in egg testosterone content observed between nests were sufficient to generate variation in physiology and behaviour remains very much a matter for speculation, as do the effects of variation in egg 17α,20β-dihydroxy-4-pregnen-3-one concentrations. However, the magnitude of inter-nest variation in egg cortisol content in some families was equivalent to the magnitude of cortisol elevation achieved in Chapter 2. I therefore propose that, if the effects in Chapter 2 were treatment-related, there could be inter-nest variation in the juvenile physiology and behaviour of the naturally-spawned offspring collected here. The next two chapters of this thesis examine the inter-nest variation in the physiology of fish reared from the eggs collected here.

In summary, this experiment has revealed that eggs deposited into different nests by a single female can vary in their steroid content. However, the lack of consistent trends in inter-nest variation, and in the influence of egg steroids on juvenile trout development, restricts my ability to predict inter-nest variation in physiology and behaviour. It is possible that length of post-ovulatory retention may affect pre-hatching egg viability, although whether any such differences could have any ecological relevance is debatable. Thus, in the determination of early individual success, spawning order could be important, but studies examining the effects of spawning order on offspring performance under natural conditions are required.

CHAPTER FIVE

DOES SPAWNING ORDER MATTER?
II. NEST-BASED VARIATION IN THE BASAL AND POST-STRESS CORTISOL CONTENT OF BROWN TROUT ALEVINS

Abstract

The physiological stress response enables animals to respond rapidly and appropriately to environmental challenges, but carries significant costs. I hypothesise that the process of emergence from the gravel is physiologically stressful for juvenile salmonids. The optimum stress response at this time will depend on the local social and physical environments, which vary in time and space. The study described in this chapter investigates whether females produce offspring with a range of stress responses as a possible strategy for dealing with environmental uncertainty. I examined basal and stressed levels of immunoreactive corticosteroid (IRC) in seven families of full-sibling alevins, and compared these levels with egg steroid content and spawning order. Both basal and post-stress IRC concentrations varied between families. Spawning order could have significant effects on alevin IRC levels both before and after a standardised confinement stressor, but trends were not consistent between or within families, and inter-nest differences were not present in all families. There were weak indications that alevin IRC levels could be related to egg steroid content. I conclude that females can produce offspring that vary in their physiological response to a stressor, and that spawning order may be an important factor in the distribution of this variation. Whether this variation impacts on energy mobilisation and survival of alevins during emergence remains to be determined.

Introduction

Prior to the complete absorption of the yolk-sac, juvenile salmonids commence feeding on exogenous materials. The onset of feeding may be concurrent with emergence from the gravel and the establishment of a territory, or juveniles may feed and move around within the gravel for a while before emerging (see Garcia de Leaniz *et al.* 2000). Changes in environment and activity levels during emergence and the onset of feeding could be physiologically stressful for young fish. Part of the generalised stress response in fish involves the activation of the hypothalamo-pituitary-interrenal (HPI) axis, a hormonal cascade that results in the synthesis of cortisol by cells in the anterior kidney (Sumpter 1997). Plasma cortisol levels are commonly elevated within 5-10 minutes of the fish being stressed (Barton & Iwama 1991), and this increase in plasma cortisol is the most commonly assessed physiological index of stress (Pottinger & Mosuwe 1994, Barton 1997). Within populations of fish (and other vertebrate taxa) the

magnitude of the cortisol response to a standardised stressor varies between individuals (Pottinger *et al.* 1992, Pottinger & Carrick 2001), and can have a genetic basis (rainbow trout: Fevolden *et al.* 1999, Pottinger & Carrick 1999). Other features of the physiological stress response are an increase in metabolism and mobilisation of energy reserves. The physiological responses of fish to stress have been extensively reviewed elsewhere (see Barton & Iwama 1991, Iwama *et al.* 1997, Schreck *et al.* 2001). Information on exactly how the different components of the stress response interact is still relatively limited, however. For example, plasma cortisol and glucose concentrations increase in response to stress, but how or whether the two are related is not well understood (Pankhurst & Van Der Kraak 1997).

Very little is known about the physiological stress response in salmonids prior to the onset of exogenous feeding. The HPI axis of late-stage alevins is activated in response to experimental handling (Barry *et al.* 1995a) and confinement (Pottinger & Mosuwe 1994), but whether experience of a stressor at this developmental stage also has metabolic consequences is unknown. For the purposes of the following argument, I hypothesise that alevins find the emergence period physiologically stressful, that energy is mobilised and cortisol produced in response to stress, that the magnitude of the stress response varies between individuals, and that the energy cost of the stress response is proportional to its magnitude.

Over a short period, the stress response is generally considered to be adaptive – supplying the fuel needed to respond to environmental change (Barton & Iwama, 1991). However, emergent salmonids often teeter on the brink of starvation (Elliott 1994), so excessive or prolonged mobilisation of energy reserves could accelerate the rate of starvation if fish do not quickly acquire a profitable feeding position. That a range of stress responses has been maintained through evolution suggests that the fitness of response varies with local environment. A fish that has acquired a profitable feeding territory soon after emergence should be better able to meet the energetic demands of a high stress response than an individual without a territory. However, if an individual emerges into a highly populated environment where feeding territories are scarce, then a lower stress response could reduce adaptive energy use under stressful conditions and increase the likelihood of survival to find an unoccupied territory as territory holders die, through predation for example. Thus the adaptive

value of the stress response could vary depending on individual energy reserves, social and physical environmental conditions, and the magnitude of the individual stress response. An inappropriate response in unfavourable conditions could have dramatic effects upon individual survival. Losses are very high in the fry dispersal phase and offspring mortality is inevitable. By producing offspring with a range of physiological characteristics a mother may be able to ensure that there is a high chance of a match between a fraction of her progeny and the available ecological niches, which are inherently unpredictable. Moreover, diversity in offspring may increase the breadth of niches that may be occupied by offspring. It is likely, therefore, that individual females can similarly increase the densities of surviving offspring by environmental manipulation of diversity, stimulated hormonally or by spawning behaviour. Finally, it has been suggested (Armstrong *et al.* 1998) that variation among the physiologies of juvenile salmonids is important for stimulating efficient dispersion from redd sites. If fish are closely matched in dominance then they may tend to fight excessively for space near the nest rather than a fraction of the progeny dispersing downstream to capitalise on vacant space.

To ensure the future of her line, it would therefore be in the interests of a female salmonid to produce offspring that vary in the magnitude of their physiological response to stress around the time of emergence, especially if individuals are inflexible in the magnitude of their response. Given the genetic basis of the stress response in many vertebrates (e.g., fish: Fevolden *et al.* 1999, Pottinger & Carrick 1999; poultry: Satterlee & Johnson 1988), it is possible that individual variation in the magnitude of the stress response within a group of offspring reflects genotypic variation. However, it is also possible that non-genetic materials in the egg at fertilisation can influence the development and activation of the HPI axis. Variable deposition of these materials could result in offspring with a range of stress responses.

In Chapter 4, it was demonstrated that egg steroid content could vary between nests spawned by a single female, hypothesised to be as a result of concentration-driven movement of steroid between eggs and ovarian fluid between spawnings. Could this degree of natural intra-family variation in egg steroid content influence the stress response of late-stage alevins? To address this question, I now investigate inter-nest variation in the basal and post-stress corticosteroid content of sibling fish about to start

exogenous feeding, and determine whether these corticosteroid measures are related to original egg steroid content.

Methods

Eggs

Eggs were collected from nests spawned by seven pairs of wild brown trout during November 2000, as described in Chapter 4. Eggs were reared at the FRS Freshwater Laboratory unit at Almondbank, Perthshire, Scotland. Nests were reared in individual containers within hatchery troughs, under standard hatchery flow and lighting conditions (described in Chapter 4).

Alevin stress response

When animals are too small to yield sufficient blood for cortisol assays, measurement of immunoreactive corticosteroid (IRC) levels in whole-body homogenates can be used to identify increased interrenal activity following environmental disturbance. However, data obtained from whole-body preparations must be interpreted with caution (see discussion by Pottinger & Mosuwe 1994), as the cortisol-specific antisera used in immunoassays may cross-react with metabolites and conjugates of cortisol in the whole body preparation that are not normally present in the plasma. Thus, rather than refer to cortisol concentrations in the alevin homogenates, I have referred to immunoreactive corticosteroid (IRC) concentrations. Despite this potential for cross-reactivity when using whole-body preparations, Pottinger & Mosuwe (1994) have demonstrated that plasma cortisol and IRC levels rise in a similar manner in response to a one-hour confinement stressor. After 8 hours of confinement, IRC levels remained elevated while plasma cortisol levels declined. Pottinger & Mosuwe (1994) suggested that these contrasting patterns of concentration change were perhaps indicative of a delay in the induction of mechanisms for the clearance of cortisol and/or its metabolites during the early phase of the response.

In this experiment, the stress response of fish from each nest was assessed by measuring IRC concentrations in alevins before and after a 1 h standardised handling and confinement stressor (10 alevins/nest at each sampling point where numbers permitted). Fish were terminally anaesthetised using benzocaine (100 $mg.L^{-1}$),

transferred to individual Eppendorf tubes and immediately frozen at –20 °C for later cortisol analyses. The standardised stressor involved the transfer of alevins into a 500 ml beaker containing 250 ml of water from the Almond river, held at ambient temperature by immersion in moving water. One beaker was used for alevins from each nest. Progeny from three families were sampled on one day, and progeny from the remaining four families were sampled 5 days later. This enabled control samples to be taken within a very short period, minimising disturbance-related elevation in basal cortisol levels resulting from the sampling of fish held in close proximity in the hatchery. Ambient water temperature was the same on the two sampling days (8 °C).

Steroid analyses

The extraction procedure was standardised to reduce possible extraction errors. Each alevin was thawed at room temperature, blotted dry and weighed to the nearest mg, then placed into a clean Eppendorf tube. Distilled water was added in the ratio 1:5 (mg:µl), and the alevin was sonicated until the tissue was completely disrupted. A sample (200 µl) of this preparation (hereafter, homogenate) was transferred to a clean Eppendorf tube, and 1 ml of ethyl acetate was added. The tube was vortex mixed for 30 s, then centrifuged at 3000 rpm for 5 min. A sample (0.85 ml) of the solvent phase was transferred to a clean Eppendorf tube, and then stored upright in a sealed container at – 20 °C until cortisol quantification.

Cortisol concentrations in the extracts were measured using radioimmunoassay, following the method of Pottinger & Carrick (2001) (see Appendix 2). Extraction efficiency was assessed by measuring the recovery of tritiated cortisol from 200 µl of homogenate as described in Appendix 1. Mean percentage recovery ± SE was 83.7 ± 0.7 % (n=6). Concentrations are expressed as $ng.g^{-1}$ alevin, and are not corrected for recovery. All samples from a single family were analysed in the same assay. Serial dilutions of alevin extracts showed that the dilution curves were parallel to the standard curve over the extract volumes used.

Statistical analyses

Basal IRC levels in some families were below the detection limit of the assay (0.75 $ng.g^{-1}$ fish). Differences between the median basal IRC levels of the different families were therefore analysed using the non-parametric Kruskal-Wallis test and post-hoc non-parametric multiple comparisons (as described in Zar 1999, p225). Post-stress IRC data were normally distributed, and inter-family differences in mean IRC concentrations were therefore analysed using a one-way ANOVA and Tukey's pairwise comparisons (family error rate of 0.05). Data from all nests were combined for these analyses.

In the absence of a test capable of dealing with non-normal data, missing nest data and unequal nest numbers in each family, the effects of nest order and the stressor on alevin IRC content could only be determined when the families were considered individually. Each family was therefore examined separately using a combination of graphics, ANOVAs and *t*-tests. Data were transformed for normality if required.

Ethical note

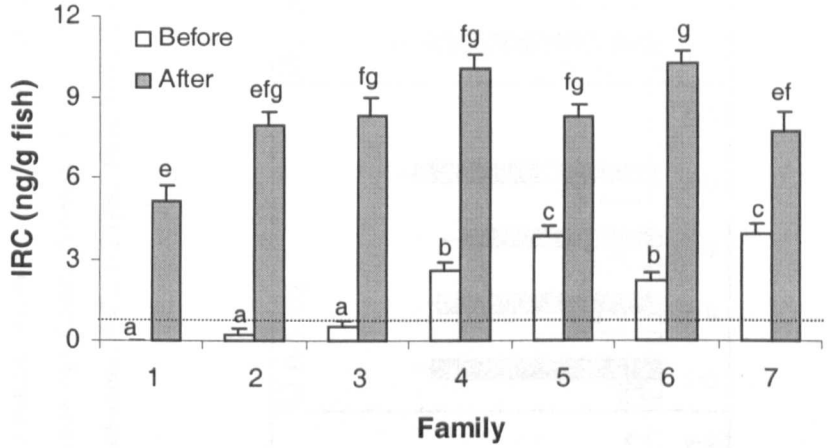
The spawning experiment was carried out under a U.K. Home Office Licence (PPL 60/2025). Eggs and larvae are not covered by the animal welfare legislation until after they commence feeding on an exogenous food source. However, they were reared under optimal hatchery conditions, and were terminally anaesthetised following the same protocol used to kill older fish.

Results

Family differences in IRC concentrations

Basal IRC concentrations varied significantly among families (Kruskal-Wallis: H=80.26, ν=6, p<0.001), with alevins from families 1-3 having lower basal IRC levels than fish from families 4-7 (Figure 5.1.). Post-stress IRC concentrations also varied significantly among families (ANOVA: $F_{6,204}$=5.92, p<0.001), but the pattern of inter-family variation was not the same as that observed for basal IRC levels (Figure 5.1.).

Figure 5.1. Mean (+ SE) IRC concentration in alevins from each family before and after a standardised stressor. Columns of the same colour with different letters are significantly different (pre-stress: Kruskal-Wallis and post-hoc comparisons; post-stress: one-way ANOVA and Tukey's test. $p<0.05$). $n\geq10$ for each family. Dashed line represents lower limit of detection.



Inter-nest variation

Basal and post-stress IRC concentrations in alevins from each nest measured are shown in Figure 5.2. Where both pre- and post-stress IRC data were available for a nest, the stressor resulted in highly significant ($p<0.001$) elevation of alevin IRC concentrations over basal levels in all but one case (family 7, nest 4). There was significant inter-nest variation in basal or post-stress alevin IRC concentrations in four of the seven families. These differences were highly significant (from $p=0.019$ to $p<0.001$). Patterns of inter-nest variation were not consistent between families. Inter-nest differences in basal and post-stress IRC concentrations within a family did not generally display the same trend.

The relationship between alevin IRC concentration, egg steroid levels and egg size

There were no apparent associations between the mean family values for alevin IRC concentrations and egg cortisol, testosterone or 17α,20β-dihydroxy-4-pregnen-3-one (17,20bP) concentrations at fertilisation (Figure 5.3.), although it must be remembered that inter-family comparisons of egg testosterone content may be erroneous due to assay limitations (see Methods, Chapter 4).

Figure 5.2. Mean (+ SE) IRC concentrations in alevins before (•) and after (▮) a standardised stressor. '?'s indicate where there were insufficient alevins to examine IRC levels. Letters above columns indicate significant inter-nest differences (*p*-values shown, *p*>0.05 considered non-significant); different letters indicate significantly different mean IRC. Dashed line indicates lower detection limit of assay (0.75 ng.g^{-1} fish). *n*=10, except for: 1-1 (post-stress, *n*=8), 1-2 (pre-stress, *n*=4), 3-3 (pre-stress, *n*=6; post-stress, *n*=8), 4-3 (pre-stress, *n*=6), 7-3 (pre-stress, *n*=9), 7-4 (pre-stress, *n*=5; post-stress, *n*=9).

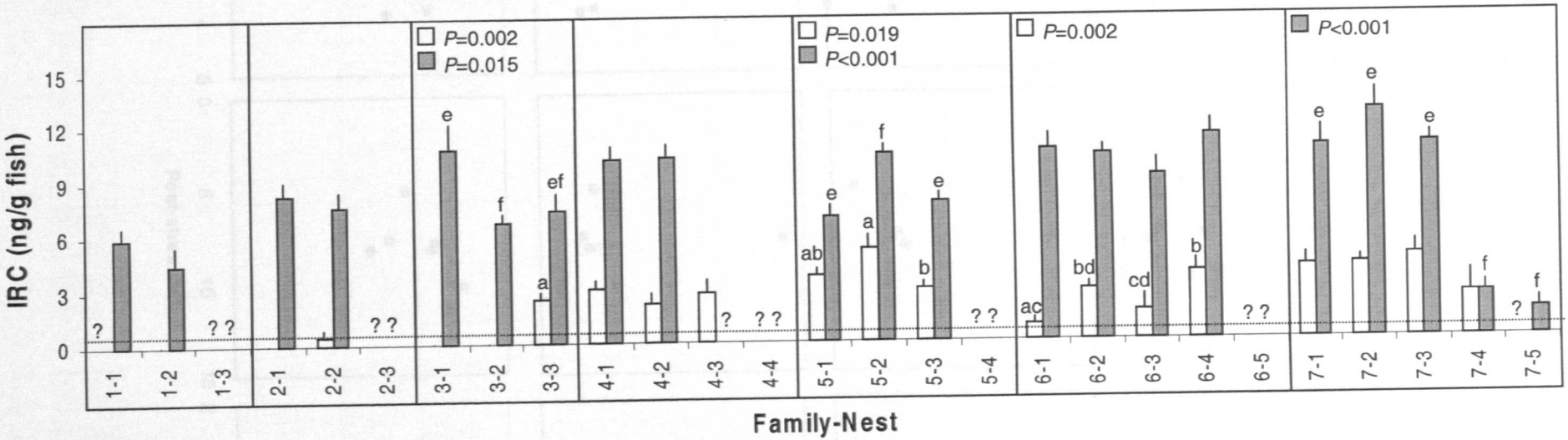
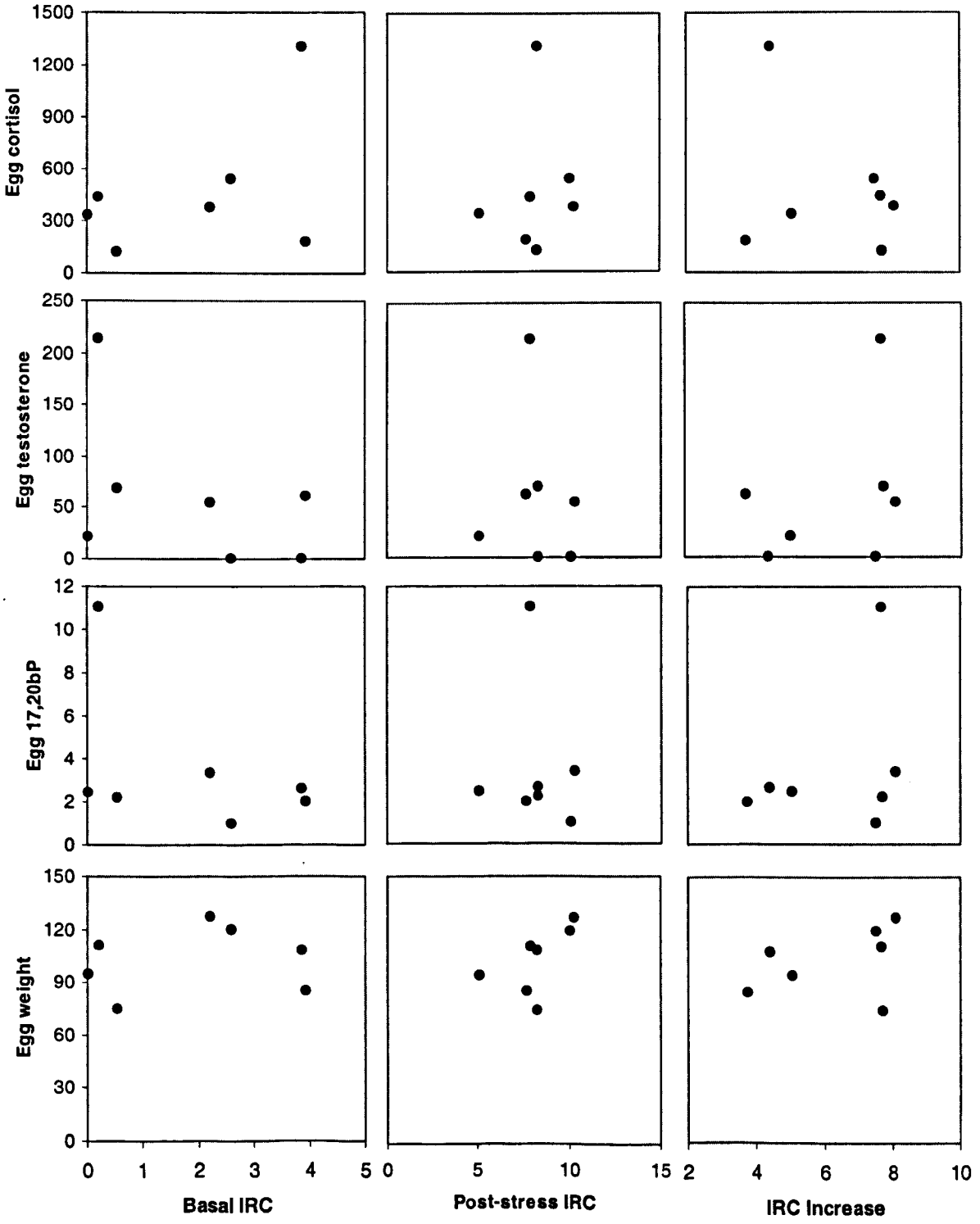
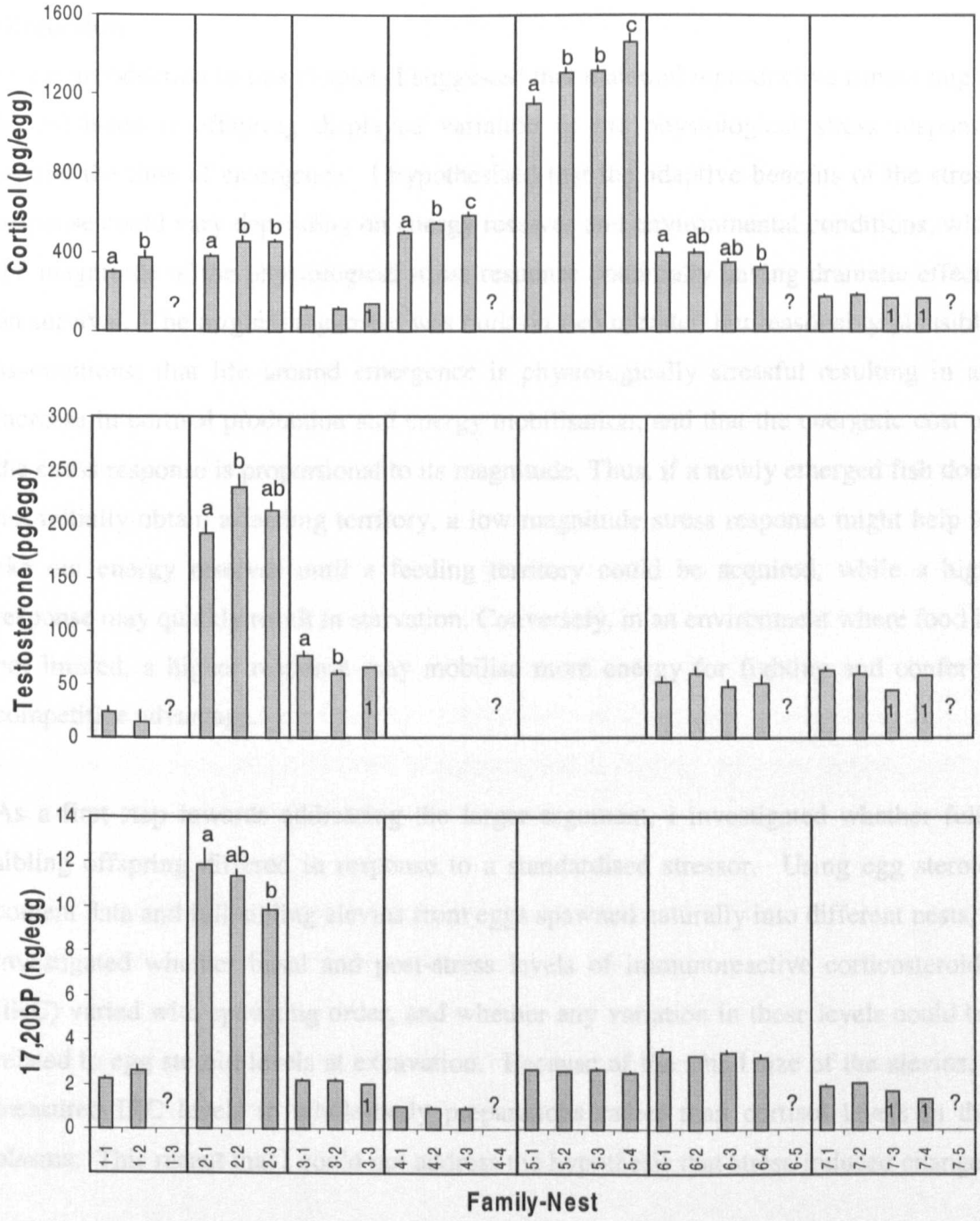


Figure 5.3. Relationships between mean family egg weight (mg) and steroid concentrations (pg.egg^{-1} for cortisol and testosterone, ng.egg^{-1} for 17,20bP) and mean family alevin IRC concentrations (ng.g^{-1} fish) before and after the standardised stressor.



Egg weight did not appear to influence either basal or stressed alevin IRC concentrations. The magnitude of response did not appear to be related to initial egg steroid concentrations or egg weight (Figure 5.3.).

Figure 5.4. Mean (+ SE) steroid content of eggs recovered from each nest spawned by seven pairs of brown trout. 17,20bP - 17α,20β-dihydroxy-4-pregnen-3-one. Note different units for 17,20bP. '?' indicates insufficient eggs to allow steroid analyses. Missing bars represent values that were below the limits of detection. Within nests from a single family, columns marked with different letters are significantly different (One-way ANOVA and Tukey's tests with a family error rate of 0.05). Except where indicated (by numbers in columns), n=6. Egg steroid data from Chapter 4.



In families where inter-nest differences in either basal or stressed alevin IRC concentrations were detected, I visually compared these patterns of variation with egg concentration data for the same families using Figures 5.2. and 5.4. (sample sizes were so small that statistical analyses of these data were impractical). Inter-nest differences in the basal IRC concentration of alevins from family 6 were (weakly) inversely related to egg cortisol content. Inter-nest variation in egg testosterone content in family 3 was mirrored by the inter-nest differences in the post-stress IRC concentration of alevins from this family. There were no other apparent associations.

Discussion

In the introduction to this chapter, I suggested that maternal reproductive fitness might be enhanced if offspring displayed variation in the physiological stress response around the time of emergence. I hypothesised that the adaptive benefits of the stress response could vary depending on energy reserves and environmental conditions, with the magnitude of the physiological stress response potentially having dramatic effects on survival. The original argument was built on two untested but reasonably plausible assumptions; that life around emergence is physiologically stressful resulting in an increase in cortisol production and energy mobilisation, and that the energetic cost of the stress response is proportional to its magnitude. Thus, if a newly emerged fish does not initially obtain a feeding territory, a low magnitude stress response might help to eke out energy reserves until a feeding territory could be acquired, while a high response may quickly result in starvation. Conversely, in an environment where food is not limited, a higher response may mobilise more energy for fighting and confer a competitive advantage.

As a first step towards addressing the larger argument, I investigated whether full-sibling offspring differed in response to a standardised stressor. Using egg steroid content data and full-sibling alevins from eggs spawned naturally into different nests, I investigated whether basal and post-stress levels of immunoreactive corticosteroids (IRC) varied with spawning order, and whether any variation in these levels could be related to egg steroid levels at excavation. Because of the small size of the alevins, I measured IRC levels in whole-body preparations rather than cortisol levels in the plasma. This meant that I could not address the hypothesis that stress-induced changes

in cortisol concentration are associated with an increase in blood glucose concentration.

This experiment has demonstrated that there are differences among families in alevin basal IRC levels, and in alevin IRC concentrations after one hour of confinement. Variation among families is consistent with previous studies that have found a genetic component to the stress response in salmonids (Fevolden *et al.* 1999, Pottinger & Carrick 1999), although inter-family variation in developmental rates (not assessed) could also contributed to the inter-family variation observed. Basal and post-stress IRC levels varied between nests of full-siblings, although trends in inter-nest variation were not consistent between basal and post-stress measurements within families. The causes and implications of these differences are not clear.

In two families, the patterns of inter-nest variation in egg steroid content and alevin IRC concentrations indicated that there may be some sort of association between the two. In one family, there was a weak inverse relationship between mean nest basal IRC levels and egg cortisol content. In a second family, the mean IRC concentration in stressed alevins was higher in fish from nests with higher testosterone concentrations. These two cases could indicate a link between alevin IRC concentrations and egg steroid levels, or between alevin IRC levels and a factor that covaries with steroid content. If these relationships are purely coincidental, then the inter-nest variation observed in this study could be due to variation in the rearing environment prior to sampling, or to inter-nest variation in some other material in the egg. Because alevins from a single family were sampled at the same time, yet were spawned approximately 12 hours apart (see Chapter 4), inter-nest variation in alevin IRC levels could also be due to alevins from different nests being sampled at slightly different developmental stages, but this is unlikely (J. Thorpe *pers. comm.*).

Inter-nest differences in the number of fish reared in each hatchery container was an obvious source of environmental variation prior to sampling. Although vigorous activity of alevins is relatively low (in the absence of external disturbance) prior to the onset of feeding (Abu-Gideiri 1966, Dill 1977, Laird & Needham 1988), it is possible that rearing density could affect basal IRC levels through social interactions. In salmonids, density can influence the frequency of social interactions (Elliott 1994), and

social experience can influence the activity of the HPI axis (Pottinger & Pickering 1992, Winberg & Lepage 1998, Sloman *et al.* 2001a). If alevins are interacting socially within the hatchery containers, then variable density could generate inter-nest variation in HPI axis activity both before and after stress.

If social interaction is not an issue, then density could still potentially affect HPI axis activity through modifications of the water quality. The flow of water was the same through all the hatchery containers. Presumably, the concentration of dissolved oxygen could be lower in containers with a higher density of alevins (i.e., earlier nests), while nitrogenous wastes could be slightly higher. Given the good supply of water to the hatchery containers and the very low mortality rates during the course of the experiment, if any differences in these factors did exist, they did not impact on survival. If water quality or social interactions were affecting inter-nest variation in IRC concentrations, then one would expect the effect to be greater in containers with more alevins. Within each family, the number of alevins reared generally decreased with spawning order (see Table 4.3., Chapter 4). Thus any effects of rearing density should be related to nest number, and inter-nest trends should be consistent between families. No consistent effects were apparent, however.

It is possible that inter-nest variation in basal and stressed IRC levels was due to inter-nest variation in egg contents other than cortisol, testosterone or 17,20bP. While Chapter 4 assessed inter-nest changes in these steroids, inter-nest variation in other materials within the egg were not considered. Given the weak indications that eggs spawned in later nests might be less viable (Chapter 4), it is possible that concentrations of maternal materials other than steroids may have changed during spawning. These materials, such as steroids, might be able to pass across the egg membrane while still within the body cavity, or they could perhaps degrade while retained in the body cavity prior to spawning and fertilisation. For example, proteins and nucleic acids of maternal origin are incorporated into the egg during development (Mommsen & Walsh 1988, Tyler & Sumpter 1996, Brooks *et al.* 1997), and they may be unstable. If these materials are in some way involved in the development of the HPI axis, then inter-nest variation in their concentrations could result in inter-nest variation in alevin IRC content both before and after the stressor.

To summarise, there was significant variation among families in mean basal and post-stress alevin IRC content, possibly due to differences in genotype or developmental stage. There was also significant variation in the basal and post-stress IRC content of full-sibling alevins spawned into separate nests in some of the families examined. I suggested a very tenuous link between this inter-nest variation and inter-nest variation in egg steroid content at excavation in two families, but the inter-nest variation is at least as likely to be due to differences in rearing environment or in maternal materials other than the three steroids measured in Chapter 4.

The magnitude of the stress response in brown trout, as in rainbow trout (Fevolden *et al.* 1999, Pottinger & Carrick 1999), is likely to have a genetic basis. Deposition of eggs into certain nests on the basis of their genotype is exceedingly unlikely. Thus the inter-nest variations in IRC concentrations reported in this chapter are almost certainly a consequence of variation in some non-genetic factor of maternal origin, or in the early rearing environment. If the former is the case, then females can influence the physiological response of their offspring to a stressor, and spawning order may be an important factor in the distribution of this variation. Whether variation in HPI axis activity impacts on energy mobilisation and survival of alevins during emergence remains to be determined.

CHAPTER SIX

DOES SPAWNING ORDER MATTER?
III. NEST-BASED VARIATION IN THE GROWTH AND METABOLISM OF JUVENILE BROWN TROUT

Abstract

Seven pairs of brown trout were allowed to spawn naturally within an artificial stream. Each pair spawned multiple times, and each nest was collected and reared separately under hatchery conditions until the eye spots became visible. Individual eyed eggs from each nest were reared in chemical and visual isolation in small containers within a large tank until two months after the onset of exogenous feeding. Egg weight, hatch date, juvenile growth and metabolic rate were measured during this time. Nest-related differences in eyed egg weight were found in one family, in time to hatching in three families, and in juvenile weight in one family. The region of the tank in which eggs were reared was found to affect time to hatching, juvenile size, metabolic rate and growth rate. Water quality decreased as water temperature climbed, resulting in high mortality and the premature termination of the experiment. The combination of position effects, high mortality, and randomised distribution of individuals among tank regions complicated data analyses and prevented the full examination of relationships between individual egg size, time to hatching, and rates of juvenile metabolism and growth. The finding that spawning order may sometimes affect hatching order and juvenile size is a completely novel result that, if not an artefact of the rearing environment, could have implications for life history processes. These results suggest that further examination of the effects of spawning order on the development and physiology of juvenile salmonids would be justified.

Introduction

During spawning, female salmonids dig and deposit eggs into nests within gravel substrata (see reviews by Fleming 1996, 1998). The digging and covering of a series of nests results in the formation of a raised area of gravel, known as a redd, running longitudinally along the stream. In the Northern Hemisphere, brown trout (*Salmo trutta*) eggs are spawned during late autumn, pass through embryonic and larval stages during winter, and emerge from the redd during spring (Elliott 1994). After hatching, the yolk-sac larvae (alevins) move down within the gravel for a period of further development accompanied by yolk-sac absorption (Huntingford 1993). Towards the end of yolk-sac absorption, the alevins begin to climb towards the stream bed, eventually emerging to commence exogenous feeding (Huntingford 1993). At this stage, fish are commonly referred to as fry (Elliott 1994). After complete absorption of

the yolk-sac and transfer to exogenous feeding, I refer to fish as juveniles. The period following emergence is a critical time in the salmonid life cycle, with high numbers of fry and juveniles often dying through starvation (Elliott 1994) or predation (Brännäs 1995).

Several studies have examined the physiology and behaviour of fry at first feeding/emergence, under both natural and simulated conditions. Considerable variation in the timing of first feeding (Metcalfe & Thorpe 1992, Garcia de Leaniz *et al.* 2000), amount of yolk remaining at emergence (Brännäs 1987, Garcia de Leaniz *et al.* 2000), site of emergence in relation to the redd (Fraser 1994, Garcia de Leaniz *et al.* 2000), metabolic rate (Metcalfe *et al.* 1995, Cutts *et al.* 1999a) and aggressiveness (Titus & Mosegaard 1991) have been detected. Many of these factors have been shown to influence survival and growth under natural and laboratory conditions (Brännäs 1987, Metcalfe & Thorpe 1992, Cutts *et al.* 1999a, Garcia de Leaniz *et al.* 2000).

As reviewed by Metcalfe (1998), there are indications that variation in the physiology of siblings at first feeding has a genetic basis in several species of salmonid. Early-feeding fry tend to be more heterozygous (Allendorf *et al.* 1983, McCarthy *et al.* 1996) and may be more likely to have a specific allele for a metabolism-related enzyme (Ferguson *et al.* 1987). But there could also be a non-genetic, maternal basis to physiological and behavioural variation among full-siblings at emergence.

The order in which eggs are spawned into nests could affect offspring performance, not simply through different spawning dates resulting in possible inter-nest timelags in offspring development, but through variation in the physical environment of the different nests within the redd (e.g., water velocity, substratum size, dissolved oxygen content, depth, temperature), or through a change in some aspect of egg quality or composition between spawnings. To the best of my knowledge, no one has examined the effects of physical variation among nests on the development or performance of salmonids from a single redd, probably for sound logistical reasons. In either a natural or artificial stream environment, it would be exceedingly difficult to assess physical characteristics of nests without disturbing their structure, and it would be equally difficult to monitor the development and emergence of offspring from different nests within a redd - assuming it were possible to keep track of spawning events in the first

place. In Chapter 4, I retrieved eggs spawned naturally by pairs of wild brown trout in an artificial stream, and reared the eggs under hatchery conditions. This approach allowed me to examine the relationships between spawning order, egg quality and composition, and offspring physiology and behaviour in the absence of nest-related environmental differences. Although this approach sacrifices direct ecological relevance for data, as the first study of its kind, any data collected will shed light on whether eggs and offspring vary between nests. If there is nest-based variation in the physiology or behaviour of offspring reared in a common environment, then individuals from different nests developing in a natural redd might also vary in these characteristics.

I have suggested in previous chapters that the steroid content of brown trout eggs may affect offspring physiology and behaviour (Chapter 2) and that there is nest-based variation in egg steroid content (Chapter 4) and may be nest-based variation in capacity to respond to stressful conditions (Chapter 5). By following the development of individual fish from egg to juvenile, I now attempt to determine if spawning order or egg steroid content influence time to hatching, and juvenile growth or metabolic rates.

Methods

Eggs

Eggs were collected from nests spawned by seven pairs of wild brown trout during November 2000, as described in Chapter 4. Eggs from each nest were reared separately under hatchery conditions at the FRS Freshwater Laboratory unit at Almondbank, Perthshire, Scotland, until 18 February 2001. At this time, 10 eyed eggs from each nest (numbers permitting), were placed in plastic bags of river water and transferred to the Glasgow University Field Station at Rowardennan, East Loch Lomondside, Scotland. A maximum of 10 eggs per nest were used because there were limits to the number of individuals that could be housed at Rowardennan. Each egg was individually weighed, then transferred to a separate container (see below) through which Loch Lomond water flowed vertically from top to bottom (flow rate through each container was approximately 2.5 $l.h^{-1}$ before the onset of feeding, 5 $l.h^{-1}$ after).

Rearing environment

The containers (Figure 6.1A.) were 10 cm sections of 10 cm diameter PVC piping sealed at the lower end with 1 mm soft mesh. Lids for each container were constructed from pipe caps (shallow, cylindrical plastic shapes with one open end). A hole (8 cm diameter) was drilled into the sealed end of each pipe cap, and this hole covered with 1 mm soft mesh. The lids were held down onto the open end of the containers by elastic bands. The containers were suspended in a layer of polystyrene held 20 cm off the base of a tank (Figure 6.1B.). This layer was vertically subdivided into seven regions. Within each region, water entered the tank at two points just above the polystyrene layer, welling up and flowing into the containers through the mesh of the lids, then passing out through the mesh base of the container. Waste left the tank via a drain in the base of the tank, and the height of water in the tank (sufficient to completely submerge the containers) was controlled by the height of the drain pipe.

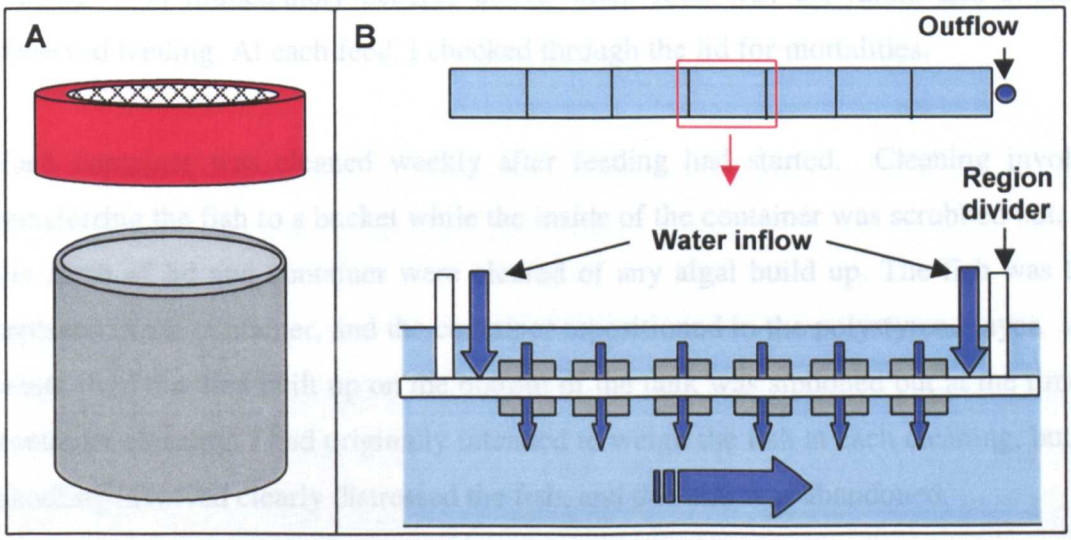


Figure 6.1. A: rearing container. B: Tank arrangement from above, and cross-section through a tank region showing water supply, and direction of water movement (blue arrows). Each tank region was*c.* 1.2 m long by 0.6 m wide, and held 45 containers. Diagram not to scale.

This vertical flow, non-recirculating system, with a separate container for each individual, ensured both visual and chemical isolation of the fish from their neighbours. I considered this important because prior social experience can affect basic behavioural and physiological characteristics of juvenile salmonids (e.g., Abbott *et al.* 1985, Rhodes & Quinn 1998). As I only had a small number of eggs from each

nest to work with, I felt that social experience could interfere with my investigation of inter-nest variation in the behaviour and physiology of full-sibling progeny. The eggs were kept in the dark until first-feeding, with the date of hatching and any mortalities being noted. Following hatching, containers were checked for mortalities once a week until the onset of feeding (see below). Offspring from all families were randomly allocated throughout the tank, regardless of nest number.

Fry and juvenile husbandry

When yolk-sac absorption was almost complete, the covers darkening the tank were removed, and the alevins were exposed to ambient light conditions. The plastic rim of the lid provided areas of shade within the containers. A slurry of water and fine commercial crumb (Trouw) made up just prior to each feed was delivered to each container manually four times daily using a 1 ml plastic syringe. Food was delivered in excess, with uneaten crumb passing out through the bottom mesh within 1 hour of feeding. Fish immediately became active when food was delivered, and could be observed feeding. At each feed, I checked through the lid for mortalities.

Each container was cleaned weekly after feeding had started. Cleaning involved transferring the fish to a bucket while the inside of the container was scrubbed out, and the mesh of lid and container were cleared of any algal build up. The fish was then replaced in the container, and the container repositioned in the polystyrene layer. Any waste food that had built up on the bottom of the tank was siphoned out at the time of container cleaning. I had originally intended to weigh the fish at each cleaning, but the handling involved clearly distressed the fish, and this aim was abandoned.

Juvenile metabolic rate and size

One to two months after first feeding (May-June 2001), the oxygen consumption ($\dot{V}O_2$) of each fish was determined. Oxygen consumption of resting fish is a direct estimate of resting metabolic rate (MR). Resting metabolic rate is exhibited by inactive fish in a postabsorptive state and reflects costs of tissue maintenance and homeostasis.

Oxygen consumption measurements involved placing individual fish into Perspex chambers through which oxygen-saturated water flowed at an adjustable rate. By measuring the depletion in oxygen between inflow and outflow under set conditions,

the rate of oxygen consumption of each fish could be assessed (see Cutts *et al.* 1998 and Appendix 3 for details of set-up and calculations). Fish were placed into the tubes 20 h prior to measuring oxygen consumption. This delay allowed fish to settle within the chambers and any oxygen debt due to initial activity to be paid off. Measurements were made at the same time each day (0900-1100) to avoid confounding effects of circadian fluctuations in consumption rates. The fish were anaesthetised and measured at the end of the metabolic rate measurements. The MR of full-siblings were measured within 24 - 48 h.

Although I had originally intended to examine the behaviour of juveniles also, the current through the containers was not sufficient to maintain good water quality as water temperatures rose, and the experiment was abandoned at the end of June. The length and weight of all surviving fish were measured at this time so that specific growth rates (SGRs) of fish between MR measurement and the end of the experiment could be calculated. The formula used to calculate SGR was:

$$100 \times [\ln (w_2) - \ln (w_1)]/\text{time}$$

where w_1 was weight at MR measurement, w_2 was weight at the end of the experiment, and time was the number of days between measurements.

Ethical note

The experiment was carried out under a U.K. Home Office Licence (PPL 60/2025). Fish were observed several times a day during the course of the experiment, and could have been removed from the experimental environment if any kind of physical distress had been observed. Indeed, the experiment was abandoned as soon as it became apparent that the water supply was insufficient to maintain water quality in the face of increasing food demands, water temperature and phytoplankton levels.

Results

Eyed egg weight and hatching

At transfer to the individual containers, there was a significant difference in egg weight between nests in one of the families (family 6: one-way ANOVA; $F_{4,42}=2.73$,

p=0.043). Eyed eggs from nest 3 were significantly larger than eyed eggs from nest 4 in this family comprising five nests (Tukey's test with a family error rate of 0.05).

Table 6.1. Results of within-family two-way ANOVAs examining the effects of spawning order (Nest) and tank position (Tank) on time to hatching.

Family	Nest	Tank
1	**$F_{2,20}$=5.79, p=0.010**	$F_{4,20}$=1.67, p=0.195
2	**$F_{2,23}$=9.96, p=0.001**	$F_{4,23}$=2.26, p=0.094
3	$F_{2,23}$=1.28, p=0.296	$F_{4,23}$=1.42, p=0.260
4	$F_{3,31}$=1.73, p=0.181	$F_{4,31}$=0.91, p=0.472
5	$F_{3,31}$=2.42, p=0.085	$F_{4,31}$=1.48, p=0.233
6	$F_{4,34}$=1.10, p=0.372	**$F_{5,34}$=3.01, p=0.023**
7	**$F_{4,39}$=2.90, p=0.034**	$F_{6,39}$=0.64, p=0.701

Hatching of the eggs began at the end of February, approximately one and a half weeks after transfer to the University Field Station, and was completed on March 22nd. This is comparable to the hatching period of eggs from the same families reared on at Almondbank (28/02/01 to 24/03/01). Hatching of eggs from different nests was fairly synchronous within families, although it appeared that eggs spawned into the first nest might hatch earlier than those spawned into later nests by the same female in some cases (Fig. 6.2.). The effects of spawning order and position within the tank (i.e., the subdivision (a.k.a. region) of the tank in which the egg was reared) on time to hatching were therefore examined using a General Linear Model (GLM, Minitab 13.30). Time to hatching for each individual was calculated as the number of days between the first observed hatching at Rowardennan (26/02/01) and the hatching of the individual. Spawning order significantly affected the mean time to hatching of eggs from three families, with eggs spawned into the first nest hatching earlier than later spawned eggs (Table 6.1.).

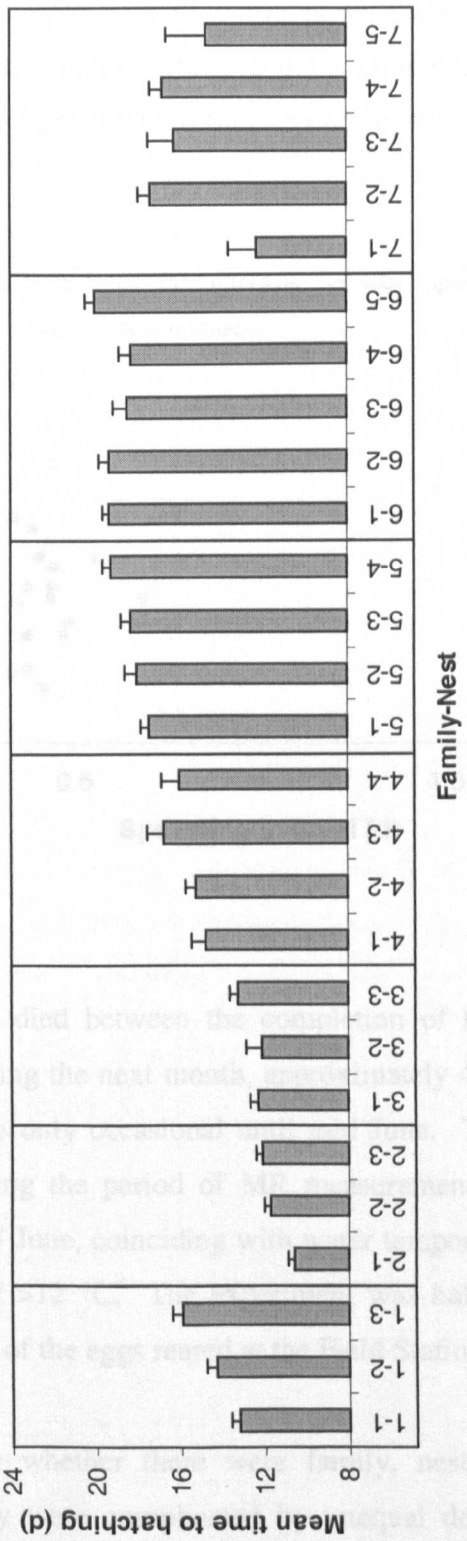
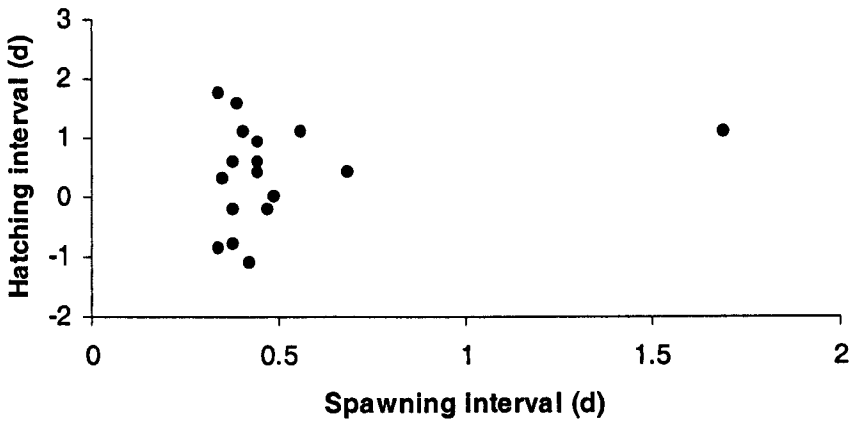


Figure 6.2. Mean (+ SE) hatching day of nests from each of seven families. Hatching day relates to the first day that hatching was recorded (26 Feb). n=10 for all nests except '7-5' (n=7).

There was a significant effect of tank position on hatching in one family (Table 6.1.), with individuals in region 1 hatching significantly earlier than individuals from regions 2 and 7. The mean hatching day (± SE) for region 1 was 17.3 (± 0.9) versus 19.9 (± 0.3) for region 2 and 20.2 (± 0.4) for region 7. There was no relationship between the length of time between spawning of consecutive nests, and the interval between hatching of eggs from the same nests (Fig 6.3.). Graphical examination of the data revealed that eyed egg weight had no influence on time to hatching within a single nest or family (Fig 6.4.).

Figure 6.3. The relationship between the intervals between spawning and mean hatching of consecutively spawned nests (all families combined).



Mortalities

Approximately 20 fish died between the completion of hatching and the onset of feeding (17 April). During the next month, approximately 40 fish died (Fig. 6.5.), but mortalities then became only occasional until mid June. The incidence of mortality was therefore low during the period of MR measurement. Forty-three deaths were recorded between 12-17 June, coinciding with water temperature rising to a consistent daytime temperature of >12 °C. The experiment was halted at this stage, with the survival of only 50.4 % of the eggs reared at the Field Station.

Attempts to determine whether there were family, nest, or tank position-related differences in mortality were complicated by unequal deposition of fish from the different families (and from different nests within families) among tank regions. Thus,

although I can report that within-family mortality ranged from 27.5 % to 70.6 %, I cannot say whether this was due to variation between families in some important physiological characteristic, due to more individuals from some families being located in less favourable tank regions, or due to individuals from some nests being less robust than others. The relationship between individual weight, MR, and mortality during the final stages of the experiment could not be examined because of very small sample sizes within families and the confounding effects of tank position.

Figure 6.4. Relationship between eyed egg weight and hatching date, by nest, for each family.

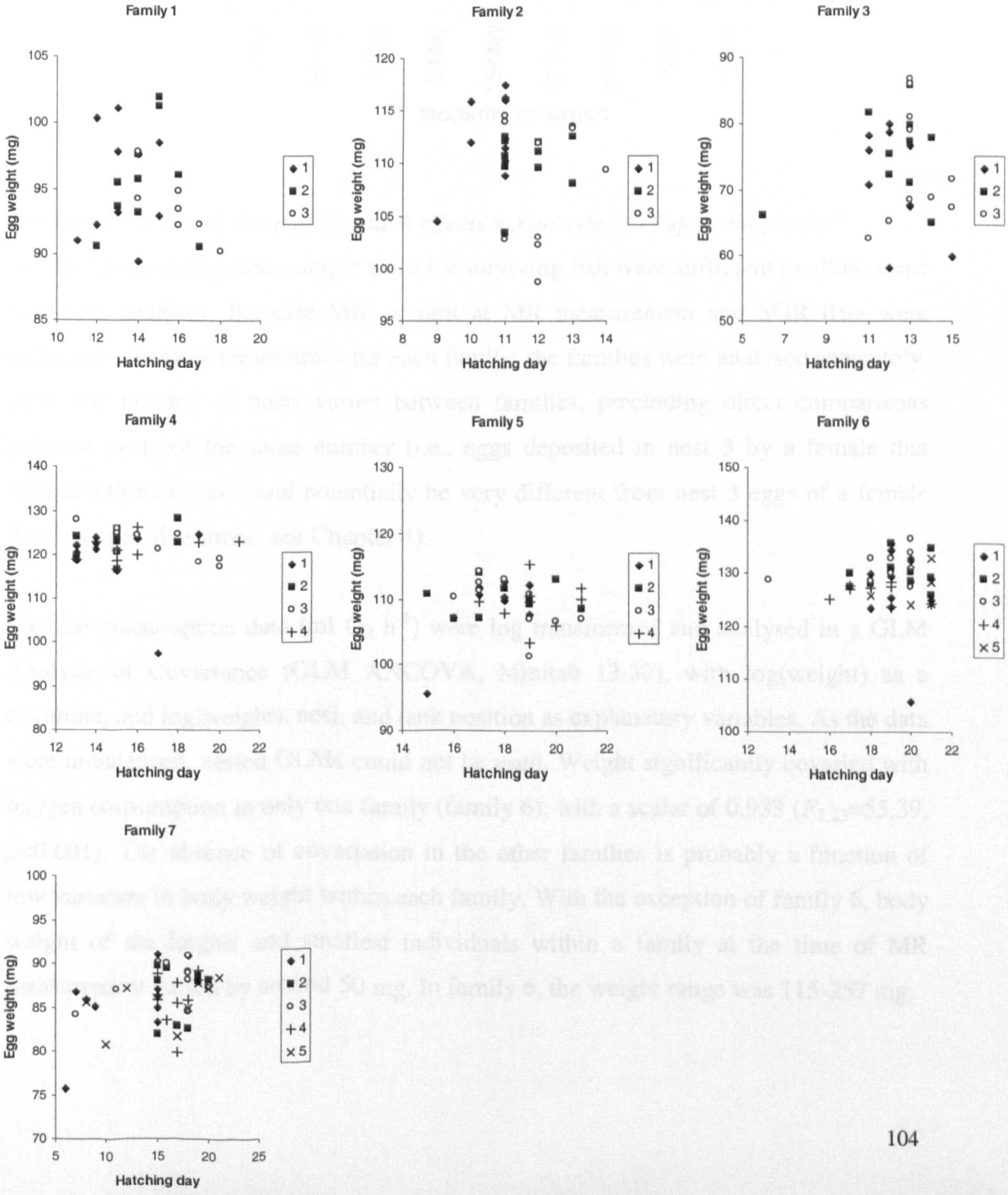
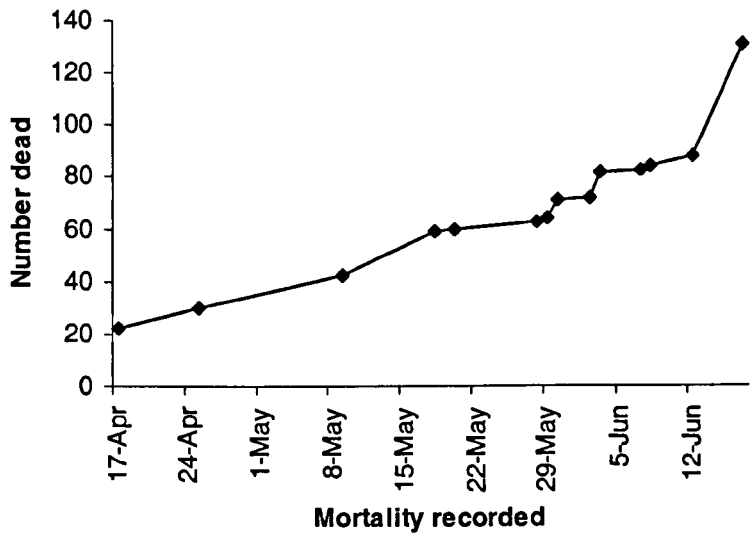


Figure 6.5. Cumulative mortalities from the onset of feeding (17 April) to the end of the experiment (17 June).



Juvenile growth and metabolism: tank effects versus effects of spawning order?

Unlike the mortality data, sample sizes for surviving fish were sufficient to allow some statistical analyses. Because MR, weight at MR measurement and SGR data were collected at/over different times for each family, the families were analysed separately. Also, the number of nests varied between families, precluding direct comparisons between nests of the same number (i.e., eggs deposited in nest 3 by a female that spawned three times could potentially be very different from nest 3 eggs of a female that spawned five times; see Chapter 4).

Oxygen consumption data (ml O_2 h^{-1}) were log transformed and analysed in a GLM Analysis of Covariance (GLM ANCOVA, Minitab 13.30), with log(weight) as a covariate, and log(weight), nest, and tank position as explanatory variables. As the data were unbalanced, nested GLMs could not be used. Weight significantly covaried with oxygen consumption in only one family (family 6), with a scalar of 0.933 ($F_{1,23}$=55.39, p<0.001). The absence of covariation in the other families is probably a function of low variation in body weight within each family. With the exception of family 6, body weight of the largest and smallest individuals within a family at the time of MR measurement varied by around 50 mg. In family 6, the weight range was 115-257 mg.

Table 6.2. Results of within-family two-way ANOVAs examining the effects of spawning order (Nest) and tank position (Tank) on the juvenile physiological variables measured. Level of significance is indicated by '*' ($p<0.05$), '**' ($p<0.01$), '***' ($p<0.001$).

	MR		**$Weight_{MR}$**		**$Weight_{final}$**		**SGR**	
Family	Nest	Tank	Nest	Tank	Nest	Tank	Nest	Tank
1	$F_{2,9}$=0.32	$F_{4,9}$=1.02	$F_{2,10}$=1.35	$F_{4,10}$=2.05	$F_{2,5}$=0.46	$F_{3,5}$=1.28	$F_{2,5}$=1.28	$F_{3,5}$=1.55
2	$F_{2,12}$=1.51	$F_{4,12}$=3.42*	$F_{2,13}$=3.95*	$F_{4,13}$=3.68*	$F_{2,10}$=7.89**	$F_{4,10}$=5.06*	$F_{2,8}$=3.63	$F_{4,8}$=15.73***
3	$F_{2,12}$=1.86	$F_{4,12}$=1.09	$F_{2,15}$=0.33	$F_{4,15}$=1.45	Small *n*	Small *n*	Small *n*	Small *n*
4	$F_{3,25}$=0.46	$F_{4,25}$=2.90*	$F_{3,28}$=1.67	$F_{4,28}$=8.01***	$F_{3,13}$=1.30	$F_{2,13}$=1.29	$F_{3,13}$=0.84	$F_{2,13}$=3.07
5	$F_{3,21}$=0.96	$F_{4,21}$=2.81	$F_{3,22}$=0.10	$F_{4,22}$=22.2***	$F_{3,9}$=0.36	$F_{3,9}$=8.06**	$F_{3,7}$=0.29	$F_{3,7}$=1.64
6	$F_{4,23}$ =2.24	$F_{4,23}$=3.86*	$F_{4,24}$ =0.73	$F_{4,24}$=4.14*	$F_{4,19}$=0.41	$F_{4,19}$=1.31	$F_{4,19}$=0.20	$F_{4,19}$=1.39
7	$F_{4,9}$ =0.64	$F_{5,9}$=2.43	$F_{4,9}$ =1.06	$F_{5,9}$=0.94	Small *n*	Small *n*	Small *n*	Small *n*

Nest number had no effect on MR, but there were significant effects ($p<0.05$) of tank position on MR data in three families (Table 6.2.).

Weight and SGR data were examined for the effects of nest number and tank position using GLM (transforming data for normality where required). As the data were unbalanced, nested GLMs could not be used. Significant effects of tank position were found in all families except 1 and 7 (sample sizes in family 3 were too small to allow tank effects to be assessed). There were significant effects ($p<0.05$) of tank position on weight at MR measurement in four families, on weight at the end of the experiment in two families, and on SGR measurements in one family (Table 6.2.). Spawning order was found to significantly affect both weight at MR measurement and final weight in one family (family 2), but did not appear to affect SGR measurements (Table 6.2.). In family 2, spawning order apparently influenced juvenile weight but, because of unequal replication within the data sets and significant 'tank effects', I was unable to statistically compare nest means to determine where the significant differences lay. However, it appeared that nest 3 individuals were smaller than those from nest 1, and possibly smaller than nest 2 individuals also.

I wished to examine relationships between egg steroid content, egg weight, hatch date, juvenile size, MR and SGR data, but the presence of 'tank effects' meant that these relationships could not be determined without first correcting the data for effects of tank position. Transforming data values for each individual to z-scores seemed the most appropriate method to use to correct the data. The z-score is the deviation of the individuals value from the mean value for that tank region for that family, expressed as a proportion of the standard deviation. However, the small sample sizes in this study resulted in extremely large standard deviations, and the z-scores probably masked any effects of spawning order even more effectively than effects of tank position did. In the absence of an effective correction procedure, I chose not to analyse these data further.

Families 1 and 7 were seemingly unaffected by rearing position within the tank. Among these families, there were a total of seven nests for which full egg steroid data were available. Mean levels of the offspring variables measured for these seven nests were regressed against mean egg steroid concentrations, but no evidence of any

concentration-dependent effects of steroids on offspring physiology were apparent ($p>0.05$).

Discussion

This chapter concludes a three-part study that examined the relationship between the order in which eggs are naturally spawned and various aspects of offspring physiology. As far as I am aware, this is the first consideration of the effects of oviposition order in salmonids, although there are numerous studies of this kind in other groups of egg-laying animals (e.g., insects: Ruohomaki *et al.* 1993; amphibians: Lyapkov 1997; reptiles: Brooks *et al.* 1991, Hays *et al.* 1993, Tucker & Janzen 1998; birds: Schwabl 1996, Lipar & Ketterson 2000). Having established that full-sibling eggs may contain significantly different amounts of steroids (Chapter 4) and that alevins developed from these eggs may vary in what could be an ecologically important physiological trait (Chapter 5), I attempted to examine the effect of spawning order on other aspects of juvenile physiology and also on behaviour. Unfortunately the experimental results were confounded by variation in the rearing environment and high juvenile mortality during the latter stages of the study. This high mortality meant that the behaviour of full-siblings from different nests could not be examined.

Limitations of the experimental design

A recurring theme throughout this chapter has been the difficulty of thoroughly analysing the data because of the effects of rearing position within the tank and small sample sizes within tank regions. Unfortunately, potential tank effects could not be avoided – I could only hope that they would be insignificant and test for them once the data were collected. As it happened, the region of the tank in which eggs were reared could have significant effects upon hatch date and all variables measured after the onset of feeding. In hindsight, I should have considered more closely the arrangement of fish within the tank, and arranged them so as to best estimate the degree of variance in the data accounted for by rearing position. Given that analyses were to be conducted separately for each family anyway, this would have allowed me to analyse the effect of spawning order while controlling for effects of rearing position. Within-family trends could have then been compared across families using a combined probablity test (Sokal & Rohlf 1995). Certainly, the wide-spread occurrence of tank effects would

suggest that rearing environment plays a far greater role in the development of individual variation in juvenile salmonids than does subtle variation in egg steroid content or spawning order. The physical differences between tank regions were not measured, but would have involved slight differences in water flow and light intensity. In my experimental set up, slight variations in water flow and the amount of light falling upon the sections could have affected water temperature, and it is well established that temperature affects the developmental and metabolic rates of salmonids (e.g., Brännäs 1987, Elliott & Hurley 1998). Despite strong tank effects, some effects of spawning order were sufficiently robust to be detected, suggesting that spawning order may have ecologically relevant affects upon juvenile salmonids.

Although very few effects of spawning order were detected, this does not mean that more did not exist. Because of the small size of the fish, weight and metabolic rate measurements were not made until 1-2 months after the onset of feeding. Mortality of juvenile salmonids under natural conditions is very high in the period following emergence, when competition for space can be intense (Elliott 1994). In the absence of social interaction and the presence of excess food, it is possible that any initial differences in juvenile physiology that might result in differential growth and survival under natural conditions would be quickly lost. It is also possible that the rearing containers used here had a significant influence on development, that could have masked nest-related differences.

In using these containers to rear the eggs individually, I traded realism for convenience. The vertical water flow was an ideal way to hold several fish in the same tank in chemical isolation, but vertical (downwards) water flow is not a condition developing fish would normally experience. The lack of gravel substrata could also have had significant effects on development and growth. Elliott and Hurley (1998) reported that brown trout alevins raised in the absence of gravel actively tried to maintain an upright position – presumably expending energy that alevins tucked into crevices in a gravel nest can reserve for growth. Indeed, alevins raised in gravel-free trays were 12-30 % smaller at emergence than alevins reared in gravel. Several other studies have reported similar effects, leading to the development and adoption of various substrata for for supporting alevins during hatchery rearing (see Laird & Needham 1988).

Effects of spawning order

At the time of transfer to the field station, eyed eggs from nest 3 of family 6 were significantly heavier than eggs from nest 4. There were no significant differences in the weight of eggs from different nests when excavated (Chapter 4), so this difference either developed during embryogenesis or was a result of sampling error. A comparison of mean egg weights at excavation with mean egg weights at transfer to the University Field Station (data not shown) revealed that egg weight did not change significantly during this phase of embryogenesis. The inter-nest variation in eyed egg weight detected here could therefore indicate that the eyed individuals were not representative of the nest they came from. If the variation in egg weight detected here is real, and indicative of developmental rate for example, then there might have been some relationship between eyed egg weight and hatching date in this family. This was not the case, however. Although there was inter-nest variation in the cortisol content of eggs from family 6 (but not in the amount of testosterone or 17α,20β-dihydroxy-4-pregnen-3-one (17,20bP); see Figure 4.3.), levels did not differ significantly between eggs from nests 3 and 4, and it is therefore unlikely that the significant differences in egg weight observed between these two nests is due to variation in egg steroid content at excavation.

As reported in Chapter 4, hatching was fairly synchronous within each family. However, individuals from the first-spawned nest hatched significantly earlier than eggs from later nests in three of the seven families. Hatching dates in these three families were unaffected by the position in which they were reared. If there were no differences in the composition or quality of eggs from a single female at spawning, I would expect eggs from all nests to hatch at about the same time. This is because water temperature was very low during spawning (*c.* 4 °C), spawning took place over a relatively short period, and embryonic development is slow at low temperatures (Brännäs 1987). Thus, an 8-12 hour interval between consecutive spawnings (Chapter 4) is unlikely to confer much of a developmental headstart to eggs spawned first. In families where the interval between spawnings is larger, or several nests are laid, then developmental differences within a family may be more noticeable, and possibly result in inter-nest variation in hatching date, and this is the case here. If the composition or quality of eggs differed between nests in such a way that developmental rate was

affected, then a different pattern might have emerged. Inter-nest variation in egg steroid content did not appear to affect the order of hatching.

As far as I am aware, a relationship between time to hatching and time to emergence of individual fry has not been reported. It is therefore difficult to know what, if any, effect an early hatching date might have on subsequent life stages. It is possible that early hatching fish are also early emergers, in which case they may be advantaged through a prior residence effect (Cutts *et al.* 1999a,b), but potentially disadvantaged through increased predation risk (Brännäs 1987) or inhospitable early spring stream conditions (Garcia de Leaniz *et al.* 2000). Early hatching might also be indicative of a faster metabolism. Metabolic rate could affect size and energy reserves at emergence, and has been linked with competitive ability (Metcalfe & Thorpe 1992, Metcalfe *et al.* 1995, Cutts *et al.* 1999a).

Analyses from family 2 indicated that spawning order could also affect juvenile size. Due to mortalities and unequal distribution of individuals from each nest among the tank regions, I was unable to statistically determine where the differences in juvenile size lay, but it appeared that individuals from nest 3 were smaller than those from nest 1 (and possibly nest 2, also). This family was characterised by a high degree of inter-nest variation in egg steroid content (Chapter 4), so it is possible that steroid levels in the eggs at excavation could have influenced early development in such a way that juvenile size was affected. Eggs from nest 3 contained significantly more cortisol than eggs from nest 1, and significantly less 17,20bP. Chapter 2 examined the effects of cortisol and testosterone, but not 17,20bP, on the physiology and behaviour of juvenile brown trout. Although the data suggested that cortisol treatment of the eggs may have influenced juvenile size in two of the five families examined, these effects may have been the result of undetected differences in rearing environment, and further speculation is therefore unwarranted without more research.

Conclusion

This experiment failed to achieve many of its aims because of unavoidable effects of rearing position within the tank, high mortality, and the unfortunate randomisation of fish placement within the tank. While I found that spawning order appeared to affect hatching order in 3 of the 7 families, any effects of early hatching remain unknown.

Whether the same patterns would be observed if eggs had been left in the redd can only be answered with further study. It may be that the effects reported here are so subtle as to have a negligible impact on survival in the face of greater hazards such as evading predation and capturing prey. Also, as the prevalence of tank effects would suggest, the effects of rearing environment on offspring development may be far greater than slight inter-nest differences in egg quality or composition. However, as the first study of its kind, this research has raised some interesting questions and avenues for future research.

CHAPTER SEVEN

THE EFFECTS OF SOCIAL STATUS ON OVARIAN DEVELOPMENT AND MATERNAL PLASMA AND EGG HORMONE LEVELS

Abstract

An experiment was conducted to examine the relationship between social status (as assessed by foraging success and relative aggressiveness) and ovarian development in small groups of maturing brown trout during vitellogenesis. Monthly physiological and behavioural measurements were made, but disease outbreaks and difficulty in identifying fish rendered much of the data unreliable. Reliable results indicated that robust social hierarchies formed under these experimental conditions, and that successful feeders were also aggressively dominant. When the fish were killed at the end of the experiment, 2-3 weeks before ovulation in this strain, larger females were more fecund. More fecund females had lower concentrations of circulating vitellogenin than less fecund females. Estimates of plasma vitellogenin concentrations were negatively associated with plasma cortisol concentrations, and positively associated with follicle weight. Larger follicles contained more testosterone. The amount of cortisol and testosterone in the developing follicles was highly variable both within and between females, with cortisol concentrations being more variable than testosterone concentrations. This experiment needs to be repeated with an effective identification regime if it is to address the original aims.

Introduction

Adverse environmental conditions (i.e., environmental stress) can impact on many aspects of fish performance (Pickering 1992). When fish are stressed, a series of neuroendocrine and endocrine responses are triggered within the fish. Initially these result in the mobilisation of energy stores to fuel the animal in its attempt to evade or overcome the challenge, but the stress response can become maladaptive if adverse conditions are prolonged (Barton & Iwama 1991, Pickering 1992, Schreck 1981 & 2000).

Part of the stress response in fish involves the activation of the hypothalmo-pituitary-interrenal (HPI) axis (Barton & Iwama 1991). This axis is a hormonal cascade that, in most fish species, results in the synthesis and release of cortisol into the blood stream (Schreck *et al.* 2001). The HPI axis of salmonid fish is activated in response to most forms of environmental stress (Donaldson 1981). Chronic elevation of plasma cortisol is known to impair many important life processes, including disease resistance, growth

and reproduction (see reviews by Barton & Iwama 1991, Pickering 1992, Balm 1997, Pankhurst & Van Der Kraak 1997, Schreck *et al.* 2001).

In salmonids, the effects of stress on ovarian development vary depending on the nature of the stressor and the stage of ovarian development at which it is applied (Schreck *et al.* 2001). Stratholt *et al.* (1997) found some evidence to suggest that application of a physical stressor (twice daily, fish were chased around the tank with a hand net for 60 s) during late ovarian development might hasten ovulation in coho salmon (*Onchorhynchus kisutch*). In a similar study on rainbow trout, exposure to a 'mild' stressor (crowding, reduction of water depth, netting, or knocking noise against the tank) during late vitellogenesis or the entire vitellogenic period also resulted in accelerated ovulation (Contreras-Sanchez *et al.* 1998). Contreras-Sanchez *et al.* (1998) and Wootton (1984) have speculated that accelerated ovulation may be a reproductive strategy when environmental conditions are not stable. If maintenance of eggs shunts energy away from homeostatic regulation, then early ovulation may be advantageous. Alternatively, there is some evidence to suggest that the onset of ovulation in unstressed salmonids is associated with an increase in cortisol levels (Bry 1985, 1989). Thus, an elevation of plasma cortisol in response to stress could pre-empt the natural rise of cortisol and trigger ovulation (Stratholt *et al.* 1997).

In contrast to these studies, Campbell *et al.* (1994) found that rainbow trout exposed to a severe stressor during late vitellogenesis had no effect on time of spawning, while complete emersion of fish in air at random intervals throughout the entire vitellogenic period delayed spawning (Campbell *et al.* 1992). Under natural conditions, the timing of ovulation will affect the timing of spawning, which may, in turn, influence the timing of emergence of offspring from the redd. As discussed in Chapters 5 and 6, the timing of emergence can influence survival and life-history processes.

Stressful experiences during ovarian development can also affect egg size, and egg size can have consequences for juvenile size and survival (Bagenal 1969, Einum & Fleming 1999). Contreras-Sanchez *et al.* (1998) reported that eggs from females stressed during late vitellogenesis were the same size as eggs from control females, but eggs from females stressed during early vitellogenesis were smaller. The mean size of eggs from fish stressed throughout the entire vitellogenic period was not statistically

different from controls, but egg size was more heterogeneous. Thus a mild stressor had very different effects on ovarian development depending on the period over which it was applied. Rainbow trout emersed at random intervals throughout the entire vitellogenic period (perhaps a more extreme stressor than those used by Contreras-Sanchez *et al.* 1998) produced small eggs (Campbell *et al.* 1992), while a severe stressor applied during late vitellogenesis also resulted in smaller eggs (Campbell *et al.* 1994). Contreras-Sanchez *et al.* (1998) explain these results in the context of trade-offs in energy allocation between body maintenance/survival and egg size/fecundity (Roff 1982). They suggest that rainbow trout stressed during early vitellogenesis put their energy into body maintenance/survival rather than egg growth, although there is scope for acclimation to the stressor and compensatory growth of oocytes following acclimation.

In the studies discussed above, plasma cortisol (Campbell *et al.* 1992 & 1994, Stratholt *et al.* 1997) or glucose (Contreras-Sanchez *et al.* 1998) concentrations were elevated in stressed fish. Campbell *et al.* (1994) also measured plasma concentrations of sex steroids and vitellogenin, and found that levels of these molecules were suppressed in stressed fish. When plasma cortisol levels in female salmonid broodstock are artificially elevated through implantation, a similar suppression of sex steroid concentrations is found (Carragher *et al.* 1989, Pottinger *et al.* 1991). Hormone receptor studies have suggested that cortisol can inhibit estradiol binding at the liver, thus suppressing vitellogenin synthesis (Pottinger & Pickering 1990, Lethimonier *et al.* 2000).

Salmonids are well known for aggressively competing for, and defending access to, resources such as food, territories, mating opportunities and spawning sites (Kalleberg 1958, Fleming 1996). Such interactions often result in the formation of social hierarchies (e.g., Adams *et al.* 1998). Social status can influence HPI axis activity in salmonids; subordinate fish often have higher levels of circulating cortisol than dominant individuals (Pottinger & Pickering 1992, Øverli *et al.* 1999a,b, Höglund *et al.* 2000, Pottinger & Carrick 2001, Sloman *et al.* 2001a). If social status affects plasma cortisol levels, and cortisol concentrations can influence concentrations of sex steroids and vitellogenin, then it is plausible to suggest that the social status of maturing females could affect ovarian development. However, to the best of my

knowledge, the effect of social stress on ovarian development in salmonids has not yet received any attention.

McCormick (1998, 1999) has demonstrated that social interactions may influence ovarian development and larval quality in the ambon damselfish, *Pomacentrus amboinensis*. Maternal plasma cortisol concentrations were elevated in response to increased competition for nesting opportunities. Increased plasma cortisol levels were associated with increased levels of cortisol in ovarian tissue and reduced larval length. The latter result was replicated when cortisol levels in fertilised eggs were artificially elevated through immersion. Social interactions may therefore influence population processes through transfer of maternal steroids to eggs.

Previous chapters have established that egg steroid content may vary considerably both within and between female brown trout, and that egg steroid content might influence the physiology and behaviour of offspring. This chapter presents a study that investigates the effects of social interactions on ovarian development and egg steroid levels, and forms the last stage in a body of work that sought to determine whether there could be steroid-mediated effects of maternal social environment on the behaviour and physiology of juvenile salmonids. This experiment considers whether small groups of maturing female brown trout held in tanks form social hierarchies and, if so, how status relates to female size and plasma concentrations of cortisol, testosterone and vitellogenin during vitellogenesis, and whether status influences egg size, egg steroid content, and rate of ovarian development.

Methods

Animals

Three-year old brown trout were purchased from the Dunsop Bridge Trout Farm, Lancashire, in May 2001. These fish had spawned as two-year olds, and were expected to spawn again in late October-early November 2001.

On arrival at the NERC Centre for Ecology and Hydrology at Lake Windermere, the fish were anaesthetised and sexed by appearance. They were then size-matched to form five groups, each containing six putative females and one putative male (outside

the spawning season it can be difficult to distinguish sex). A male was included in each group for two reasons. Firstly, to provide some of the pheromonal cues females normally encounter during maturation and, secondly, to provide a focus for inter-female competition during the final stages of ovarian development. If female social status is related to competitive ability, then it is important for the aims of this experiment to limit valuable resources throughout the entire period of development. Initially competition will be for food, but female appetite is diminished in the final stages of maturation (Pickering & Christie 1981). Females of some salmonid species will compete for male company when males are in short supply (van den Berghe & Gross 1989).

Fish within each group were size-matched to allow me to determine whether any variation in maternal behaviour and physiology was due to differences in fish size, or to differences in social status. As fish become dominant, they may take more food than others and become larger. By grading the groups so they had different mean sizes (Table 7.1.), data from fish of the same size, but not necessarily the same rank, could be compared between groups. Each group was stocked into a separate tank (see below).

Table 7.1. Mean weight of fish (n=7) in each group at the start of the experiment.

Tank	Mean weight ± SE (g)
1	1093 ± 32
2	857 ± 34
3	1039 ± 87
4	1114 ± 52
5	1427 ± 41

While anaesthetised, each fish in a group received a unique combination of Alcian blue marks on the fins, and a brightly coloured plastic bead was attached to the adipose fin using suture thread.

Mortalities

Soon after arrival, the fish showed signs of both furunculosis and fungal infections. While most infections cleared up, during June severely affected fish were killed and replaced with spare fish. Replacement fish were not size-matched to their group. Two fish failed to recover from anaesthetic during the August sample, but were not replaced. During October, several fish in one tank succumbed to fungal infections. At the close of the experiment, two tanks contained seven fish while the remaining three tanks contained three, five and six fish.

Tanks and routine husbandry

Each group was held outside in a circular tank (1500 l capacity) with a water depth of 30 cm. The tanks were supplied with a constant exchange of water pumped from Lake Windermere (30 $l.min^{-1}$), with water draining from the tank via a grill in the centre of the tank floor. The tanks were covered by lids of plastic mesh, a section of which was covered with black polythene to provide shade for the fish.

Tanks were cleaned during the monthly sampling (described below), to remove algae from walls and floor and thus facilitate behavioural observations. Fish were fed three times a week by dropping pellets (Trouw, standard 60) through the mesh lids at a set point. Waste food was washed out of the tank in the outflow.

Sampling

Each group of fish was observed for one day a month, and blood sampled and measured the following day.

(a) Behavioural observations

At the start of the day, one half of the lid was removed, and the fish then allowed to settle for half an hour. Ten minutes before starting to feed the fish, I took up a sitting position at the side of the tank, ensuring that I cast no shade into the tank. I fed the fish by slowly flipping pellets into the centre of the tank at a rate of approximately 6 $pellets.minute^{-1}$. The pellets sank downwards to the drain, clearing the tank within 5-8 seconds if not captured. I deemed the fish to be uninterested in feeding at that observation point if I had offered 50 pellets at the start of a feeding period without eliciting a feeding response. If pellets were taken, I offered them until either 200

pellets had been captured, or 10 consecutive pellets had gone to waste, whichever occurred first. I recorded the identity of the fish taking each pellet. Following feeding, I observed the fish for 15 minutes, during which time I recorded the identities of fish winning and losing agonistic encounters. Agonistic encounters included bites and chases (Keenleyside & Yamamoto 1962, Øverli *et al.* 1999a), and a win recorded when one of these actions resulted in evasive behaviour (swimming away, retreat to the tank floor with fins lowered, or swimming at the surface of the tank) in the recipient, or when the recipient of one of these behaviours responded aggressively and caused the initiator to display evasive behaviour. I repeated the feed and post-feed observations in the afternoon.

Pilot studies showed there were very few aggressive interactions between fish outside of feeding or immediate post-feeding periods.

I totalled the number of pellets captured by each fish over the two feeds, and ranked these totals within each group to give each fish a feeding rank for each month. The fish that had captured the greatest number of pellets within its group was ranked 1, the fish that captured the least was ranked 7. I also totalled the number of agonistic interactions won and lost by each fish for the two post-feed observation periods, and calculated an aggressive index (AI) for each fish (AI = [number of wins/total number of interactions]) for each month. I then ranked the fish on the basis of AI magnitude. The fish within the group that had the highest AI was defined as the aggressively dominant individual within that group, and assigned a rank of 1, while I gave a rank of 7 to the least successful, subordinate, fish. I do not refer to fish of intermediate rank in the results section, so 'subordinate' always refers to the least aggressive fish in a group.

(b) Physiological assessment

To avoid initiating a physiological (or behavioural) stress response, the fish were 'stealth' anaesthetised. This involved leaving the lid in place, raising the outflow pipe, then slowly squirting 180 ml of 2-phenoxyethanol (approx. 1:2000 dilution, Sigma P1126) into the tank inflow before closing the inflow valve. By squirting the anaesthetic into the inflow, the chemical was thoroughly mixed within the tank. The fish became unconscious within 5 minutes of the anaesthetic being administered, and

were then swiftly netted from the tank into a large bin filled with dilute anaesthetic solution (1:10000 dilution of 2-phenoxyethanol). Fish were taken one at a time from this bin, blood sampled, weight and length measured, and then placed in a recovery bucket before being returned to the tank (emptied, cleaned and refilled during sampling). If necessary, beads (but not after the July sampling) and Alcian blue marks were reapplied.

Blood (0.6-1.0 ml) was taken from Cuverian sinus (a sinus underneath the opercular flap) using heparinised 1 ml syringes and 25G needles. Blood was transferred into 1.5 ml Eppendorf tubes and held on ice until all samples had been collected. The samples were then centrifuged at 3000 rpm for 10 minutes at 4 °C, and the plasma stored in 200 µl aliquots at –20 °C until being analysed for steroid and phosphate content (see below).

Terminal sampling

This strain of brown trout ovulates and spawns during the last week of October and first two weeks of November. To compare ovarian development between fish of different social status, I decided to kill and sample all fish on the same day in mid-October, approximately 2-3 weeks prior to ovulation. Fish were observed for a day as described above. On the following day, all fish were terminally anaesthetised and coshed. Fish were blood sampled, weight and length measured, then dissected open along the ventral midline from anus to pectoral girdle. The ovaries were removed and weighed. Follicle samples were taken for hormone analyses, stage of development, and size measures. Follicles taken for hormone analyses were teased free of surrounding connective tissue, weighed, and then frozen until extraction. Follicle size was estimated by wet weight. Previous pilot studies (Chapter 3) have shown that follicle diameter can be affected by the process of removing follicles from connective tissue (can squash the follicle), and that follicle diameter is positively associated with follicle weight. To provide an estimate of fecundity, a subsection of the ovary was weighed, and the number of follicles in this sample counted. This is more accurate than dividing ovary weight by average follicle weight, as the ovary subsection contains connective tissue. Twenty-two females survived to the end of the experiment, several putative females proving to be male

Female and follicle weight and fecundity data were normally distributed. Associations between these variables were examined using Pearson's correlations. End point steroid data from follicles and plasma were not normally distributed, and were therefore rank-transformed for investigation using Spearman's rank correlations. All analyses were two-tailed.

Steroid analyses

Steroids were extracted from plasma and follicles as described in earlier chapters and Appendix 1. Extraction efficiencies are given in Appendix 1. Testosterone and cortisol concentrations in plasma were measured following the radioimmunoassay protocol described by Pottinger & Carrick (2001). This protocol is described in previous chapters and in Appendix 2. Steroid concentrations are not corrected for extraction efficiency.

Alkali-labile phosphate (ALP) assay

This assay measures protein-bound phosphate in plasma samples, and provides a good estimate of vitellogenin levels in the blood (vitellogenin being a phospholipoprotein). Phosphate was extracted from plasma following the method of Wallace & Jared (1968), and quantified using the method of Stephens (1963). I added 5 ml of 10 % trichloroacetic acid to 25-100 µl of freshly thawed plasma, and stored the samples overnight at 4 °C to precipitate all plasma proteins. The following morning, I centrifuged the tubes at 2000 rpm for 10 minutes. After discarding the supernatant, I mixed the protein precipitates with 5 ml of 5 % Trichloroacetic acid to ensure complete precipitation of proteins, and incubated the tubes at 90 °C for 30 minutes before centrifugation at 2000 rpm for 10 minutes. I then washed the precipitates with successive organic washes: 100 % ethanol (10 minutes at 60 °C), chloroform:ether:ethanol (1:2:2), acetone, and ether. I left the washed precipitates to dry in the fume cupboard for 45-60 minutes, before partially hydrolysing them in 1 ml of 2N NaOH for 15 minutes at 100 °C. The samples were cooled and neutralised with 1 ml 2N HCl, before I added 2.2 ml of distilled water and 800 µl of freshly prepared mixed reagent (5N sulphuric acid: sodium molybdate:L-ascorbic acid:antimony potassium oxide tartrate in the ratio 5:2:2:1) to each tube. After mixing, I allowed the tubes to rest at room temperature for at least 10 minutes to develop colour. I then

measured absorbance in a spectrophotometer at 880 nm. Phosphate standards (KH_2PO_4) covered the range 0-2.8 $\mu g.tube^{-1}$. Further details of reagents are given in Appendix 8.

Results

Fish identification

Beads tied to the adipose fin were repeatedly displaced from most fish, but were not reattached after July. Chi-squared tests of the July data showed that bead retention was not related to aggression-based social status (χ^2=1.55, df=4 (ranks 3 & 4 and 5 & 6 pooled), p>0.05). The attachment site appeared to be susceptible to fungal infection and I therefore preferred not to continually retag because of the risk of infection and the potential for subsequent effects of infection on growth and ovarian development.

Fish that retained their beads were readily identifiable during behavioural and physiological sampling. During behavioural observations, fish without beads were given nominal identifications based on their physical appearance, but it was not always possible to positively match the appearance of the swimming fish to their appearance during blood sampling. This difficulty was exacerbated by the original size-matching of fish in each tank. It was very difficult to identify distinguishing physical marks when there were several healthy fish of a similar size in a tank. Thus behavioural data for fish without beads could not be matched to physiological samples.

Fish identification during physiological sampling was further complicated by the loss of Alcian blue marks on the fins. Fading of marks, erosion and splitting of fins, and scarring of fins from fungal infections made it difficult to positively identify Alcian blue marks. Where Alcian blue marks were clear, they were refreshed during monthly sampling, but in some cases it was not possible to confidently identify marks. Some of these 'unmarked' fish could be identified by physical abnormalities, but others could not. Again, size-matching made it difficult to distinguish between fish.

Erring on the side of caution, I only examined data from fish that I was able to identify with complete confidence (i.e., fish that had a bead only). This meant that I had

limited data on behaviour and physiology for a few fish, and that I was generally unable to track individuals throughout the course of ovarian development. However, the data collected at the termination of the experiment allowed me to examine associations between maternal size and blood parameters and egg size, number and steroid content 2-3 weeks prior to ovulation.

Relationship between aggression and feeding success

In June, at the peak of infection, the fish were not feeding or interacting with one another. The July and August behavioural data, however, were very good – fish were generally feeding well, and there were many agonistic interactions after feeding. The water clarity was poor in September, making it impossible to observe the fish, while in October the fish showed little interest in feeding or fighting.

There was a significant positive relationship between individual ranks for aggression and feeding success in both July and August (Spearman's rank correlations, n=35: r_s=0.388 and r_s=0.586 respectively, p<0.05). These results indicate that aggressively successful fish were also successful feeders. It was not possible to match data for each individual between July and August, but ranks of the individuals with beads could be compared. Aggression ranks did not change between months (Spearman's rank correlation, n=6, r_s=0.955, p=0.003), but feeding ranks were less stable over time (r_s=0.265, p=0.612).

Maternal physiology during ovarian development: effects of social status?

Three individuals had beads at all sampling points, two of which were in the same group. The weight, length, condition factor, and plasma concentrations of cortisol, testosterone and ALP for the three beaded individuals are illustrated in Figure 7.1. Fishes 30 and 32, in the same social group, started the experiment at the same weight. Fish 30 dominated both feeding and agonistic encounters in this tank, while fish 32 was the subordinate individual in the group. Thus, the loss of weight by fish 32 over the course of the experiment may be due to reduced food intake as a consequence of low social status. While plasma testosterone and ALP concentrations were very similar for these two fish, the cortisol profiles varied considerably. Cortisol concentrations in the plasma of fish 32 were elevated above those of fish 30, although remaining within the range normally accepted for 'unstressed' female brown trout at

this stage of maturation (approx. 5 $ng.ml^{-1}$ plasma; Pottinger *et al.* 1991). Follicular cortisol and testosterone concentrations and follicle weight did not differ between these two fish at the end of the experiment (Table 7.2.; *t*-tests, $p>0.05$).

Fig. 7.1. Weight, length, condition factor (100 x weight/length^3), and plasma concentrations of cortisol, testosterone and ALP in beaded females sampled throughout the experiment. Fish 24 (+), fish 30 (diamond), fish 32 (square). Fish 24 and 30 were dominant within their groups, fish 32 was subordinate.

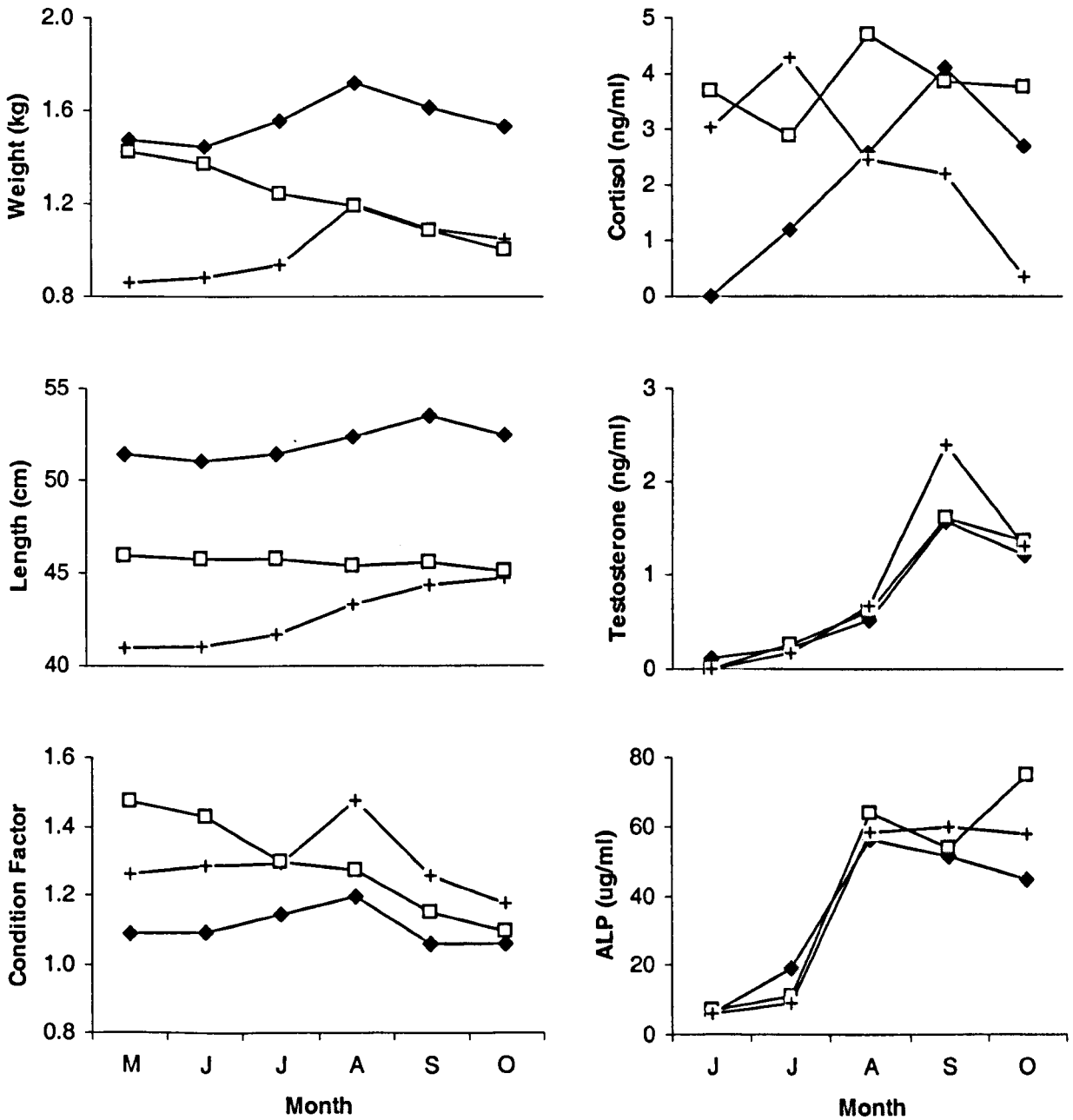


Table 7.2. Follicle characteristics (mean ± SE) and social status of the three beaded fish. Different superscripts indicate a significant difference between means (α=0.05).

	Fish		
	24	30	32
Fish rank	Dominant	Dominant	Subordinate
Follicle weight (mg)	53.42 ± 0.29^{a}	56.35 ± 0.51^{b}	55.63 ± 0.30^{b}
Follicle testosterone concentration (ng/g)	3.59 ± 0.36^{a}	1.83 ± 0.35^{b}	2.31 ± 0.42^{b}
Follicle cortisol concentration (ng/g)	15.38 ± 5.81^{a}	1.48 ± 0.30^{b}	0.83 ± 0.35^{b}

Fish 24 was the third fish to retain a bead throughout the experiment. This fish was initially much smaller than fishes 30 or 32, but gained weight to end the experiment at the same size as fish 32. Follicles from fish 24 were significantly smaller than those of the other two beaded fish (Table 7.2.; One-way ANOVA, $F_{2,143}$=16.29, p<0.001, Tukey's pairwise comparisons with family error rate of 0.05), but follicle concentrations of both testosterone and cortisol were significantly higher (Table 7.2.; $F_{2,17}$=34.41, p<0.001 and $F_{2,17}$=11.01, p=0.001, respectively). Levels of plasma testosterone were very similar in fish 30 and 32 at the September sampling, but were much higher in fish 24 at this time. The pattern of weight and length change (but not condition factor) in fish 24 paralleled that of fish 30. Like fish 30, fish 24 was the aggressively dominant individual within its tank. It is therefore possible that this pattern of growth typifies aggressively dominant individuals under these experimental conditions. Obviously, with a sample of only three fish, conclusions cannot be drawn, and inferences only cautiously made.

Terminal sampling

At the end of the experiment, larger females were more fecund (r=0.925, p<0.001), but there was no association (p>0.05) between female weight and follicle weight, or between follicle weight and fecundity. Concentrations of ALP in maternal plasma were negatively related to plasma concentrations of cortisol (r_s=-0.480, p<0.05), so females with higher plasma cortisol levels had lower levels of circulating ALP. There were no other significant correlations between plasma and follicle concentrations of ALP and steroids. However, maternal ALP concentrations were negatively correlated with

steroids. However, maternal ALP concentrations were negatively correlated with maternal fecundity (r_s=-0.467, p<0.05), so that more fecund females had lower levels of circulating ALP. Despite ALP concentrations correlating with both plasma cortisol levels and fecundity, there was no association between fecundity and plasma cortisol concentrations. Follicle weight was positively correlated with the concentration of testosterone within the follicle (r_s=0.598, p<0.01). All other possible associations were non-significant (p>0.05), although there were indications that follicle weight may also be positively associated with maternal plasma ALP concentrations (r_s=0.427, critical value at α=0.05 is 0.428).

The variation in maternal and follicular content among females is shown in Table 7.3., as is the degree of variation in follicular steroid content within females. The mean follicular testosterone concentration was less than that of cortisol, and follicular testosterone content varied less both between and within females than did cortisol. Within maternal plasma, levels of testosterone were again generally much lower than the concentration of cortisol, and variation in testosterone concentrations among females was also much lower. The degree of variation among females in plasma ALP concentrations was of the same magnitude as the testosterone data.

Table 7.3. Variation in follicular steroid concentrations and in ALP and steroid concentrations in maternal plasma at the termination of the experiment. Follicular testosterone and cortisol coefficient of variation (CV) values refer to the within-female variation in follicle steroid content.

	Mean	Min	Max	CV
Follicles				
Testosterone (ng.g^{-1} tissue)	1.97	0.00	3.59	-
Testosterone CV	15.6 %	6.7 %	31.0 %	-
Cortisol (ng.g^{-1} tissue)	5.44	0.35	15.38	-
Cortisol CV	60.7 %	15.5 %	192.6 %	-
Plasma				
Testosterone (ng.ml^{-1})	1.11	0.05	1.56	23.6 %
Cortisol (ng.ml^{-1})	7.60	0.34	64.99	226.5 %
ALP (µg.ml^{-1})	66.12	6.17	136.68	34.0 %

Discussion

I had intended this experiment to examine whether social interactions influenced ovarian development and egg steroid levels in brown trout. While I designed the study very carefully, its success was dependent on a tagging system that would allow me to trace the behaviour and physiology of individual fish throughout the course of the experiment. When my tagging system failed, it became impossible to address the original aims of the experiment. However, given the outbreaks of *Furunculosis* and *Saprolegnia* during the early part of the experiment, it is doubtful whether I could have conclusively attributed any effects to social interactions.

The immune and endocrine systems are closely linked (see Balm 1997). Exposure to pathogens can alter plasma steroid concentrations, reproductive function and growth. Thus, the *Furunculosis* and *Saprolegnia* outbreaks in the first months of the study may have affected plasma steroid levels and ovarian development in my trout. From the behavioural observations made in June and size measurements taken in June and July, infection certainly appeared to affect feed intake and growth.

In retrospect, implanting the fish with passive integrated transponder (PIT) or visible implant (VI) tags at the start of the experiment would have ensured that I was able to trace the physiological samples of each fish throughout the study. Although I considered these tagging approaches, the equipment required was not readily available, and neither PIT nor VI tags would have helped with the behavioural observations. Under some experimental conditions, PIT tags can be very useful in tracing the movements of individual fish (e.g., Huntingford *et al.* 1998), but this would not have been sensible in the Windermere environment.

A reliable, relatively non-invasive method of visibly marking fish within stock tanks so that an external observer can identify them has yet to be developed. Prior to the start of this experiment, I tested several different marking methods to see what marks would be most visible during behavioural observations. Dye and paint marks (of varying colours) on the body and fins did not stand out or persist, whereas the beads allowed for very rapid and positive identification. Although visible tags could have been more securely attached through the dorsal musculature, neither I nor my collaborators at Windermere were prepared to use such an invasive tagging method.

Attachment of beads through the adipose fin, with Alcian Blue back up marks on the fins seemed like the best solution. Unfortunately, this was not the case.

Using the reliable data, it appears that feeding and aggressive dominance were positively associated within our social groups. This result is consistent with previous studies of the relationship between aggressiveness and foraging success in fish (Li & Brocksen 1977, Fausch 1984, Metcalfe 1986, Metcalfe *et al.* 1989, Huntingford *et al.* 1990, Johnsson *et al.* 1996, McCarthy *et al.* 1999). Under defensible conditions (e.g., point source of food delivery, temporally predictable delivery), individual ranks of aggressiveness and foraging success are generally positively correlated, although individual ranks for the two behaviours may not match exactly (Adams *et al.* 1998, Bailey *et al.* 2000). If food availability is more temporally or spatially unpredictable, or if food is delivered to excess, then the relationship between dominance and feeding success begins to break down (McCarthy *et al.* 1992, Winberg *et al.* 1993, Martin-Smith & Armstrong 2002).

My data also indicated that social status (as defined by aggression, but not by feeding success) was maintained between the July and August observations. Thus the experimental design was successful in that small groups of maturing brown trout held under these conditions formed robust aggression-based social hierarchies. There could be several reasons as to why the feeding ranks of individuals were not consistent between these observations. Fish were recovering from infection, which may have affected motivation to feed. Individuals may also have differed in levels of hunger at the two sampling points, affecting their rankings.

A closer consideration of the three individuals that retained their beads throughout the course of the study showed that the two dominant individuals similar growth and steroid profiles during the course of ovarian development. Body weight of the two dominant individuals increased to August, after which they lost weight again until the end of the experiment. Aggressively-dominant fish continued to put energy into somatic growth throughout vitellogenesis, as evidenced by the increase in forklength (although length measures were affected by fin erosion towards the end of the study period). In contrast, the subordinate individual lost weight throughout the study, and did not increase in length. By repeating this experiment with a more successful tagging

regime, it may be possible to determine whether these patterns of growth are characteristic of fish of low and high social status.

I was able to examine relationships between characteristics of mother and follicles using the data collected at the termination of the experiment. The number of developing follicles increased with female weight, a relationship that has been reported several times for salmonids (Elliott 1984, Fleming 1996), but female weight did not influence follicle size. This is in contrast to the work of Elliott (1984) that demonstrated a positive relationship between maternal body length and the weight of brown trout eggs. Indeed, increasing egg size with maternal size appears to be a relationship widespread among the salmonids (see review by Fleming 1996). However, I killed the fish during vitellogenesis, and the follicles are likely to have increased in size as vitellogenesis progressed. Thus, by ovulation, a relationship between female size and egg weight may have developed.

Fecundity was inversely related to levels of alkali-labile protein-bound phosphate (ALP) in the female plasma. ALP is accepted as a good estimate of vitellogenin levels in the circulation of fish (Gagne & Blaise 2000). Thus, my result indicates that there is less circulating vitellogenin in females with more developing follicles. More fecund females may have been taking up vitellogenin into the eggs at a faster rate than less fecund females, resulting in a lower concentration of vitellogenin in circulation. Alternatively, given that more fecund females were found to be larger (and will therefore have a greater total blood volume), the absolute amount of vitellogenin in circulation in larger females could be the same (or even higher) than in less fecund females.

ALP concentration in female plasma was also related to levels of circulating cortisol; levels of circulating ALP were lower in females with higher plasma concentrations of cortisol. This result is consistent with earlier studies that have shown a suppression of plasma vitellogenin levels in response to both stress-induced (Campbell *et al.* 1994) and artificial (Carragher *et al.* 1989, Pottinger *et al.* 1991) elevation of cortisol in trout. It may be, therefore, that elevated plasma cortisol levels suppressed vitellogenin synthesis in my fish. Plasma steroid and ALP concentrations reported here are similar to those collected for the same strain of brown trout held in tanks of the same

dimensions in groups of 10-20 throughout ovarian development (TG Pottinger, unpublished data).

Follicle weight at the end of the experiment was correlated with egg testosterone concentration; larger follicles contained more testosterone. Testosterone is synthesised in the follicle layer surrounding the developing oocyte (Tyler & Sumpter 1996). Larger oocytes will have a larger surface area and the follicle layer will probably contain more testosterone-producing cells. Thus there is increased opportunity for testosterone uptake into the oocyte.

Plasma and follicular concentrations of cortisol varied more than did concentrations of testosterone or ALP. While consistent with the findings of Chapter 3, the follicular CVs described here were much higher for both steroids. This possibly reflects the difference in developmental stage between follicles – in Chapter 3, oocytes had finished vitellogenesis and were within hours of being ovulated from the follicles. Here, oocytes would not be ovulated for another 2-3 weeks. Variation in follicle steroid content might decrease over this time.

This experiment, while unsuccessful in its original intent, has provided data that indicate that aggression-based social hierarchies are formed under these experimental conditions. At the end of the experiment, larger females were more fecund. Estimates of plasma vitellogenin concentrations were negatively associated with plasma cortisol concentrations, and positively associated with follicle weight. Larger follicles contained more testosterone. The amount of cortisol and testosterone in the developing follicles was highly variable both within and between females, with cortisol concentrations being more variable than testosterone concentrations. If this experiment were repeated with an effective tagging regime, it is likely that the results would address the question of how social status influences ovarian development. Ideally, any such study would extend to consider the physiology and behaviour of the offspring also.

General Discussion

Maternal steroids in salmonid eggs: a cause of phenotypic variation?

Phenotypic variation is the basis of selection (Darwin 1859). In salmonids, individual variation in traits such as metabolic rate, time of emergence from the nest and juvenile size can affect competitive ability (reviewed by Metcalfe 1998, Sloman & Armstrong 2002). The ability to compete for or monopolise resources can influence not only the survival and reproductive success of an individual, but also the timing of major life events such as when to mature or migrate to sea (see reviews by Thorpe 1987, Armstrong *et al.* 1998, Thorpe *et al.* 1998, Metcalfe 1998). While there is a genetic basis to many important physiological traits (Ferguson *et al.* 1987, Heath *et al.* 1993, McCarthy *et al.* 1996, Pottinger & Carrick 1999) that can influence competitive ability of juvenile salmonids (Metcalfe 1998, Pottinger & Carrick 2001, Sloman & Armstrong 2002), the actual expression of that genotype will be influenced by the environment of the fish. Mothers can influence the nature of the early environment by modifying the size or composition of the egg (including hormone content), or (under natural nesting/spawning conditions) by the deposition of eggs into slightly different physical environments (reviewed in Mousseau & Fox 1998). The primary aim of this thesis was to examine the possibility that variation in the hormonal composition of salmonid eggs could influence important developmental processes. Specifically, I investigated the following:

1. Can the amount of steroid in the egg at fertilisation affect the phenotype of offspring?
2. If so, do egg steroid levels actually vary between and within females?
3. If natural variation exists within the eggs of a single female, does this variation influence offspring phenotype?
4. How does maternal social environment influence ovarian development and the amounts of steroids deposited in developing eggs?

1. *Can steroids in the eggs affect offspring phenotype?*

In Chapter 2, I experimentally elevated concentrations of testosterone or cortisol in the fertilised eggs of five brown trout. Several months after the onset of feeding, I detected treatment effects on juvenile size in three families, but effects were not consistent. I also found differences in behaviour in one family, and differences in resting metabolic rate in one family. There was no effect of treatment on sexual differentiation. Given

the inconsistency in results, it was impossible to determine whether the effects observed were due to treatment, or to undetected differences in the rearing environment of different groups (i.e., *tank effects*). However, elevated steroid levels were within the physiological range for these eggs, so (under these rearing conditions) the amount of certain steroids in the egg at fertilisation could potentially affect ecologically important aspects of juvenile physiology and behaviour.

2. *Do egg steroid levels vary within and between females?*

In order for maternal steroids to be responsible for phenotypic variation within and between offspring from different females, there needs to be natural variation in egg steroid concentrations. It was apparent from the data presented in each chapter that egg steroid content varied between females, so the possibility certainly exists that maternal steroids could contribute to phenotypic variation between families.

In Chapter 3, I established that there was considerable variation in egg size and cortisol content among eggs from single females and hypothesised that this variation was related to varying proximity of developing oocytes to blood vessels. The variation detected in this chapter was mainly assessed before ovulation. Post-ovulatory variation in egg steroid content was also detected both between and within females. Prior to spawning, cortisol concentrations were higher in eggs lying in the anterior part of the body cavity, although only four females were sampled, and these results must be treated with caution (Chapter 3). When females were allowed to spawn naturally (Chapter 4), inter-nest variation in cortisol content was detected in the eggs of five of the seven females examined, but the differences were inconsistent – in four cases, the amount of cortisol in the eggs increased over consecutive spawnings, but decreased between the nests of one female. Inter-nest variation in egg testosterone content was detected in eggs from two of the seven females, but the pattern of variation was not consistent. Finally, concentrations of the progesterone derivative 17α,20β-dihydroxy-4-pregnen-3-one varied between the nests of one of the seven females. I hypothesised that inter-nest variation arises after ovulation, as eggs rest in ovarian fluid in the body cavity awaiting deposition.

Inter-nest differences in egg testosterone content were less than the experimental elevations described in Chapter 2, so whether the differences in egg testosterone content observed between nests were sufficient to generate variation in physiology and behaviour remains very much a matter for speculation, as does the effect of variation in egg 17α,20β-dihydroxy-4-pregnen-3-one concentrations. However, inter-nest variation in egg cortisol content in some families was equivalent in magnitude to the cortisol elevation reported in Chapter 2. If treatment was responsible for the effects observed in Chapter 2, it is therefore possible that the inter-nest variation in egg cortisol content observed in Chapter 4 could generate inter-nest variation in the juvenile physiology and behaviour of the naturally-spawned offspring if reared under similar hatchery conditions.

3. *Do spawning order and natural variation in egg steroid content result in phenotypic variation?*

In Chapter 4, I identified a weak relationship between spawning order and egg viability prior to hatching, with eggs spawned first being more viable than those spawned later in five of the seven families examined. These differences in viability were unrelated to egg steroid content. Although I discovered nest-based variation in the pre- and post-stress levels of immunoreactive corticosteroid (IRC) of alevins from some families just prior to complete yolk-sac absorption (Chapter 5), there was no consistent relationship between spawning order and alevin IRC concentrations. Nor was there any good evidence for a relationship between inter-nest differences in egg steroid content and nest-based differences in alevin IRC concentrations.

By rearing offspring from each nest to two months after the onset of feeding (Chapter 6), I was able to investigate the effects of spawning order at other developmental stages. Nest-related differences in eyed egg weight were found in one of the seven families, in time to hatching in three families, and in juvenile weight in one family, but no effects of spawning order on juvenile metabolic rate or specific growth rate were detected. Analyses of the effects of spawning order on offspring were confounded by tank effects and high mortality. Nest-related differences in time to hatching were consistent with the order in which the nests were spawned, but differences in egg and juvenile size did not increase or decrease with the order of spawning. Nest-related

differences in offspring did not appear to be associated with inter-nest variation in egg steroid content.

So, does spawning order influence offspring phenotype? In some cases, but this is not consistent, and tank effects could be confounding the results. Does natural variation in egg steroid content influence offspring phenotype? There is no evidence to support such a conclusion. Egg steroid levels may be associated with the development of some of the inter-nest variation described in Chapters 5 and 6, but further research is required to fully elucidate their involvement. What is obvious from Chapter 6, is that variation in early rearing environment can have profound effects on individual phenotype, but that maternal effects (i.e., the deposition of eggs into different nests) may also contribute to phenotypic variation.

4. *Does maternal social environment influence ovarian development and egg steroid content?*

In Chapter 7, I constructed social groups of maturing brown trout and attempted to track individual behavioural and physiological status during vitellogenesis. This study is the first to consider the relationship between social status and ovarian development in salmonids. Unfortunately, disease and difficulty identifying the fish rendered much of the data collected unreliable. However, there were sufficient reliable data to determine that groups of maturing brown trout do form stable aggression-based hierarchies, that aggressive dominance is associated with feeding success, but that feeding ranks are not stable over time. There was a negative relationship between fecundity and plasma vitellogenin concentrations, and between plasma cortisol and vitellogenin concentrations, but no evidence of a relationship between plasma and follicular steroid concentrations at the time the fish were killed (2-3 weeks prior to ovulation). Follicle weight and testosterone concentrations were positively correlated, and there was weak evidence for a positive association between follicle weight and plasma vitellogenin levels. The nature of any relationships between plasma and egg steroid concentrations and maternal social status remain unclear.

In summary, this thesis describes a series of reasonably ambitious but well-designed experiments (within fiscal, logistical and temporal constraints) that have produced

some novel and complex information on the extent of variation in the steroid content of eggs both within and between females. Whether these steroids have a role in the development of phenotypic variation in juvenile physiology and behaviour remains unclear because of potential (Chapters 2 and 5) and identifiable (Chapter 6) effects of variation in the rearing environment. My results have also indicated possible effects of spawning order, but interpretation of data must remain cautious because of inconsistent results and the prevalence of tanks effects. Further research is required to clarify the situation, but it is clear that phenotypic variation in juvenile salmonids is the product of complex and subtle interactions between genotype and early environment. This thesis will alert fish biologists to a rich variety of possible effects that are both interesting in themselves and important for experimental design. Throughout this thesis, I have assumed that brown trout are interchangeable with other salmonids that display nesting behaviour and a period of freshwater, non-schooling, behaviour, but this may not be the case.

Relationships to other work

In fish, this research represents only the second investigation into the potential importance of maternal steroids on the development of progeny in an ecological (as opposed to larvaculture) context. Like me, McCormick (1998, 1999) found a high degree of inter-family variation in response to treatment when he examined the effects of elevation in egg cortisol or testosterone on development of Ambon damselfish. He suggested that some of this variation in response could be related to differences in egg steroid concentrations. This was not the case in the study described in Chapter 2, as elevations in egg steroid concentrations could increase or decrease the level of a physiological measure (cf: controls) in different families. However, my findings may be a reflection of tank effects rather than treatment.

The effects of variation in egg steroid content on the development of progeny have been best studied in the birds. Here the picture appears to be more distinct, with patterns and effects of variation in steroid concentrations being consistent within a species. However, between different taxonomic groups of birds, the same patterns of steroid differences can have very different effects on progeny. For example, the amount of maternal testosterone deposited into eggs increases with laying order in the canary (*Serinus canaria*) and the American kestrel (*Falco sparverius*). While

increased testosterone appears to be adaptive in the canary by conferring a competitive advantage to later hatching chicks (Schwabl 1996), increased testosterone delayed hatching and reduced nestling growth and survival rates in the kestrel (Sockman & Schwabl 2000).

Although I was unable to draw any conclusions from my study of maternal social status due to disease and tag loss, it would have been fascinating to compare the results of such a study with similar research in birds. The amount of testosterone deposited into the eggs of black-headed gulls (*Larus ridibundus* L.) is higher in smaller eggs (Groothuis & Schwabl 2002). It was hypothesised that increased androgen levels in smaller eggs may compensate for lower nutritional quality of eggs. Androgen levels were also higher in eggs laid in areas where there is high maternal competition for nesting sites, and it was suggested that these levels reflect maternal plasma levels of androgens, elevated as a consequence of age, aggressiveness and social stimulation. Whittingham & Schwabl (2002) reported that levels of maternal testosterone in the eggs of tree swallows (*Tachycineta bicolor*) varied with levels of female aggression before and during egg laying. If elevated androgen levels are associated with high social status in female salmonids, the eggs of dominant females could also have a high testosterone content. This remains to be examined.

The ecological relevance of variation in egg steroid concentrations

"...the physiological and ecological literature is replete with adaptive reasons for variation in particular ... traits. However, rarely have these explanations been the subject of rigorous testing." (Spicer & Gaston 1999, p. 96)

Throughout this thesis, I have hypothesised that variation in concentrations of steroids in the egg at fertilisation could generate variation in the behaviour and physiology of full-sibling brown trout under natural conditions, but how likely is this? Assuming the effects witnessed under controlled rearing conditions were not an artefact of undetected differences in rearing environment, then the effects of egg steroid content were subtle and inconsistent. Are such effects really going to have a significant influence on development in an unstable and unpredictable environment? One answer is 'no'; another is that the importance of steroid concentrations during early

development may be amplified by variation in environmental conditions so that effects in the wild could be far greater than were detected here.

Regardless, success at emergence is often critical to survival and dispersal in the wild (Elliott 1994, Armstrong *et al.* 1998), so any factor that influences characteristics that can determine early competitive ability and survival is important. At emergence into an uncertain environment, the chances of at least some offspring surviving to pass on a female's genetic material may be increased if offspring of a single female display a range of behaviours and physiological characteristics. If egg steroid levels can influence characters that affect survival at emergence, and if egg steroid levels can vary between nests, then the performance of offspring from different nests emerging into the same environment could also vary. If such effects occur (and this remains to be determined), I suggest that it is an accidental mechanism arising as a consequence of unavoidable changes in maternal physiology during spawning, rather than an effect that has evolved to increase female fitness. Endocrine systems are highly conserved between vertebrate groups, and I suggest that only very strong evolutionary forces would be able to alter the regulation of those systems. A slight heterogeneous advantage to the mother does not seem sufficiently powerful to have such an effect.

Implications for hatchery practices

One of the challenges in salmonid aquaculture is to understand why egg quality (e.g., fertilisation and survival rates) varies between females (Bromage *et al.* 1992, Brooks *et al.* 1997, Schreck *et al.* 2001). My research has provided no evidence to suggest that egg steroid content influences egg viability. However, the (extremely) limited results from Chapter 7 (where I examined groups of maturing trout for evidence of social structures) suggest that social status may affect plasma steroid levels and patterns of energy allocation during ovarian development. Thus, social rank may affect egg size and steroid content.

Future research directions

The results described here indicate some tantalising possibilities that may have important implications for salmonid ecology and aquaculture. However, given the variability of results and other problems encountered during these studies, it is premature to speculate on the role of spawning order and egg steroid contents in the

development of phenotypic variation without first gathering more information. It would therefore be sensible to repeat my experiments with a greater degree of replication, and to follow up some new avenues of investigation. By repeating and expanding the experiments detailed in this thesis, it may be possible to demonstrate whether the effects of treatment and spawning order reported here are real, and not artefacts of extremely subtle variation in the pre-hatching or juvenile rearing environment. It is also possible that egg steroid content could affect physiological or behavioural traits that I have not considered. Further examination of the effects of social status on ovarian development is needed to identify the importance of social stress in broodstock maintenance.

The experimental elevation of egg steroid content described in Chapter 2 needs to be repeated with replication of each treatment within each female. Eggs from each female need to be exposed to a range of concentration doses of each steroid, to see if effects of treatment are strongly concentration-dependent within sibling eggs. Molecular studies to assess the effects of steroid concentrations on gene expression at different stages of development are also required, as are investigations into other compounds within the yolk that steroids could be interacting with. Recent work into the actions of egg thyroid hormones during early development have revealed that maternal thyroid hormone receptor RNA is present in the yolk of salmonid, halibut and sea bream eggs at fertilisation (Sweeney *et al.* 2002). Concentrations of steroid receptors and binding proteins could significantly influence the effects of steroids during early development. There may also be complex and subtle interactions between the genotype of individual eggs and the amount of steroid present. The expression of many genes is controlled by hormone-responsive elements (Sweeney *et al.* 2002). If full-siblings have slightly different genotypes, then the number and sensitivity of these elements could vary between eggs, influencing the action of steroids and the effect of steroid concentration.

At the other end of the scale, the ecological relevance of egg steroid levels and spawning order will only be understood if the survival and performance of fish from eggs of known steroid content and different nests are assessed under natural conditions. No simple matter.

Implications for experimental design

The research described in this thesis is characterised by a high degree of inter-family variation in the physiological characteristics assessed. These findings have wider implications for experimental design. Several studies that have investigated factors determining individual variation in the physiology and behaviour of juvenile salmonids have used a single family of full-sibling individuals (e.g., the importance of metabolic rate: Metcalfe *et al.* 1995, Cutts *et al.* 1998), as a way of reducing experimental noise created by genetic variation. A similar approach has been taken by researchers in many areas of biology (Spicer & Gaston 1999). This is a sensible approach to understanding biological processes, but I suggest that it is important to replicate these studies at the family level also, to ensure that results are consistent within a population.

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Appendix 1 Efficiency and accuracy of the procedure used for extracting steroid hormones from eggs, homogenised fish, plasma and ovarian fluid.

Background

This thesis has focused on variation in egg hormone levels, and thus it is important that the procedure used to extract hormones from the eggs is as accurate as possible. Here I assessed the reliability of the egg extraction procedure at two stages – at homogenisation, and at solvent extraction of the steroids. In addition, I assessed the efficiency with which steroids were extracted from fish homogenates, plasma and ovarian fluid.

Homogenisation

The homogenisation of egg tissue was standardised. Eggs had been frozen in Eppendorf tubes in pools weighing approximately 200 mg. The eggs were thawed, and the membranes then disrupted within the tubes using 20 strikes of a mounted needle. The mounted needle was then wiped 10 times on the rim of the tube before 200 µl of 0.1 M NaOH was added, and the tube vortex mixed for 30 seconds. This vortex mixing appeared to successfully collect yolk at the rim of the tube. Between samples, the needle was rinsed once in each of 0.1M NaOH, distilled water and ethanol, then wiped dry.

To test the reliability of this homogenisation procedure, the loss of material during homogenisation was measured. This was carried out by measuring the egg weight, and the 'eggs + tube' weight before and after homogenisation. The loss of weight through homogenisation was calculated in absolute terms (mg of sample) and as a percentage of the sample weight. The results are shown in Table 1. Very little material was lost, and the amount lost was relatively consistent. This method was therefore considered to be reliable.

Ideally, I would have rinsed the needle in NaOH following homogenisation and added the rinsate to the homogenate, but this was not practical. Egg yolk is remarkably sticky, and a large volume of NaOH would have been required to properly rinse the needle. The total volume of NaOH used in the extraction process had to be minimised if eggs were not to be transferred to larger tubes (a ratio of NaOH:solvent during extraction of at least 1:5 is preferable, and the Eppendorf tubes used had a maximum capacity of 1.5 ml). Using large volume tubes was not a practical option given the time and cost involved, and limited access to centrifuges able to accommodate larger tubes.

Table 1 Loss of egg material during homogenisation.

	Weight loss (mg)	% Sample loss
Mean ± 1 s.e.m.	5 ± <1	2.21 ± 0.97
Minimum	2	1.05
Maximum	13	5.02
n	27	27

Solvent extraction efficiency

To examine the efficiency of solvent extraction of steroids from egg homogenates, tritiated cortisol or testosterone were added to the NaOH, and 200 µl of these tritiated solutions were added to the eggs after homogenisation. After the standard vortex mixing and 1 h at room temperature, the steroids were extracted using ethyl acetate as described in the various chapters. The extracted solvent was aspirated off into scintillation vials and, following the addition of liquid scintillation fluid, counted. The recovered counts were then expressed as a percentage of the total counts initially added to the homogenates.

To assess extraction efficiency of fish homogenates (Chapter 5), plasma and ovarian fluid, 10 µl aliquots of tritiated solutions of cortisol or testosterone in 100 % ethanol were evaporated into empty Eppendorf tubes. 200 µl of homogenate or plasma/fluid was then added to these tubes, refrigerated for 1 h, then frozen at –20 °C for 12 hours

before being thawed and extracted with ethyl acetate as described in the various chapters. The amount of recovered tritium was expressed as a percentage of the amount initially added to each Eppendorf tube. Results are given in Table 2.

Table 2 Efficiency of solvent extraction of steroids from eggs, plasma, ovarian fluid and fish homogenates. Values represent the mean ± 1 standard error (n=6).

Sample	Cortisol Recovery (%)	Testosterone Recovery (%)
Chapter 2 eggs	56.5 ± 0.4	84.7 ± 0.5
Chapter 3 eggs	59.5 ± 0.5	
Chapter 4,5,6 eggs	56.7 ± 0.9	77.5 ± 1.4
Chapter 7 eggs	60.5 ± 0.7	79.4 ± 0.9
Plasma	97.6 ± 0.3	96.5 ± 0.4
Ovarian fluid	95.7 ± 0.5	98.6 ± 0.7
Fish homogenates	83.7 ± 0.7	

The recovery of testosterone from egg homogenates was higher than that of cortisol, while recovery of the two steroids from plasma and ovarian fluid samples were equivalent. Extraction efficiency of the third steroid measured in chapters 4 and 7 of this thesis, 17α,20β-dihydroxy-4-pregnen-3-one (17,20bP), was not assessed due to a shortage of tritiated 17,20bP.

Appendix 2 Steroid radioimmunoassay protocol

Radioimmunoassay is a competitive binding assay that allows determination of the amount of a substance in tissue or fluid. The basic approach is to first extract the substance (in this case, steroid hormone) from the biological material, and to then measure the amount of steroid in the extract. Extraction selectively harvests steroids from other materials that may interfere with antibody binding during the assay. I have used the cortisol radioimmunoassay method described by Pottinger & Carrick (2001) for the quantification of cortisol in egg, plasma and ovarian fluid samples. By simply exchanging the cortisol antibodies, inert steroid, and radioactive steroids used in the protocol described below, I have modified the protocol for the quantification of testosterone and 17a,20b-dihydroxy-4-pregnen-3-one.

Cortisol

Plasma samples were extracted by vortex mixing with ethyl acetate (1:5, plasma:ethyl acetate). After centrifugation, an aliquot of the extract (20-150 • l) was transferred to a 3.5 ml polypropylene assay tube. A standard curve was constructed by adding to a series of assay tubes, oin duplicate, aliquots of ethyl acetate containing between 12.5 and 800 pg of inert cortisol (Sigma Aldrich). Blank tubes received ethyl acetate alone. A 50 • l aliquot of ethyl acetate containing 20,000 dpm of [1,2,6,7-^{3}H]cortisol (Amersham Pharmacia Biotech; 60 Ci.mmol^{-1}) was added to all the tubes and the solvent was evaporated under a vacuum. A 200 • l aliquot of anti-cortisol antibody (IgG-F-2; IgG Corp; 1:600) in phosphate-buffered saline (PBS; Sigma) containing bovine serum albumin (RIA grade; Sigma; 0.1 %) was added to each tube (except two nonspecific binding tubes that contained [^{3}H]cortisol only) and the tubes were incubated overnight at 4 °C. After incubation, racks containing the assay tubes were placed on ice and a 100 • l aliquot of chilled, stirred, dextran-coated charcoal in PBS (1.0 % activated charcoal; 0.2 % dextran) was added to each tube. After vortex mixing, the tubes were incubated on ice for 5 min before being spun (3000 g at 4 °C for 10 min). A 200 • l aliquot of the supernatant was added to 4.5 ml of scintillant (Ecoscint A; National Diagnostics) in a vial, mixed by inversion, and counted under standard ^{3}H conditions.

Cortisol concentrations in the unknown samples were calculated from the equation of a 3-parameter hyperbolic function fitted to a plot of the percentage of [^{3}H]cortisol bound against picograms of inert cortisol (Sigmaplot; SPSS Science). Sensitivity (minimal detection limit) of the assay was 12.5 pg.tube^{-1}. The inter-assay coefficients of variation for low (25 pg.tube^{-1}) and high (400 pg.tube^{-1}) samples were 8.5 and 8.0 % (n=8) and the corresponding intra-assay coefficients of variation were 5.4 and 5.1 % (n=8).

Testosterone

Sensitivity (minimal detection limit) of the assay was 12.5 pg.tube^{-1}. The inter-assay coefficients of variation for low (25 pg.tube^{-1}) and high (400 pg.tube^{-1}) samples were 10.3 and 5.8 % (n=8) and the corresponding intra-assay coefficients of variation were 5.7 and 5.3 % (n=8).

17α,20β-dihydroxy-4-pregnen-3-one

Sensitivity (minimal detection limit) of the assay was 5 pg.tube^{-1}. The inter-assay coefficients of variation for low (25 pg.tube^{-1}) and high (400 pg.tube^{-1}) samples were 11.0 and 6.8 % (n=6) and the corresponding intra-assay coefficients of variation were 5.7 and 5.0 % (n=8).

The intra-fish variation in steroid concentrations reported in this thesis is higher than the intra-assay variation of these assays (all samples from a single fish processed in a single assay).

Appendix 3 Respirometry methodology

Resting metabolic rates of fish were estimated in Chapters 2 and 6 using methodology previously described by Cutts *et al.* (1998).

Oxygen consumption rates of juvenile trout were measured by placing individual fish in Perspex respirometry chambers through which water continuously flowed at a known rate. Water temperature was noted in order to estimate the capacitance of oxygen in the water (βWO_2, ml $O_2.L^{-1}$) during a series of oxygen consumption measurements. Water temperature was kept constant at a temperature representative of the ambient throughout each 24-hour trial (see below), and was kept fully oxygen-saturated by means of an airstone. The water then flowed by gravity from a header tank through the respirometry chambers.

A rack of 20 such chambers allowed the oxygen consumption rates of 20 trout to be measured on the same day. The fish were placed in the chambers overnight, and measurements commenced 20 h later, by which time they had settled and evacuated their guts (Higgins and Talbot 1985), and any oxygen debt due to differences in activity prior to placing in the chambers would have been paid off. Faeces washed through the outflow. Pilot experiments had indicated a stable oxygen consumption after this period of acclimation. Moreover, the fish were kept in darkness to minimise activity, and the low flow rates (mean flow rate = 0.025 ± 0.001 $l.h^{-1}$) through the chambers precluded any active swimming against a current. Oxygen consumption ($\dot{V}O_2$, ml $O_2.h^{-1}$) was measured by first injecting 0.5 ml of 100 % oxygen saturated water from the header tank (representative of the water flowing into each chamber) into an oxygen electrode, and then injecting a 0.5 ml sample of water flowing out of an occupied chamber and noting the percentage reduction in oxygen saturation from 100 %. The percentage reduction in oxygen concentration was converted to oxygen consumption ($\dot{V}O_2$, ml $O_2.h^{-1}$) using the equation:

$$\dot{V}O_2 = V_w.\Delta C_wO_2 \qquad (1)$$

Where V_w is the flow rate of water through the respirometry chamber ($l.h^{-1}$; measured by collecting the water outflow from each tube into a beaker over a measured time period (a minimum of 2 minutes) and weighing it) and ΔC_wO_2 is the difference in the oxygen concentration between the inflow and outflow water ((% reduction/100)βWO_2, ml $O_2.l^{-1}$; where βWO_2 is the capacitance of oxygen in the water).

This procedure was repeated three times for each fish (with a minimum of 30 minutes between measurements), and the average value was taken for each fish. The entire procedure was identical for each fish, allowing a comparable measure of standard metabolic rate for individuals. For a fuller account of the respirometry methodology, see Cutts *et al.* (1998).

To calculate whether a fish had a relatively high or low respiration rate for its size, the regression line of oxygen consumption (ml $O_2.h^{-1}$) versus weight (g) on a double natural logarithmic scale was used to calculate the expected metabolic rate of a fish of a particular weight. This was then compared with its observed metabolic rate (ml $O_2.h^{-1}$). Absolute rather than weight-specific values were used, since weight-specific values usually decrease with increasing body size, i.e. larger fish respire less on a per gram basis than smaller fish. The difference between a fish's observed and expected resting metabolic rate (rMR, measured in ml $O_2.h^{-1}$) was then calculated by subtracting the expected value from the observed; a positive value therefore indicated that a fish had a higher than expected oxygen consumption rate for its size while a negative value indicated a relatively low consumption rate.

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Eye colour in juvenile Atlantic salmon: effects of social status, aggression and foraging success

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ABSTRACT

In salmonids, social subordination results in darkening of the skin and eyes, which may act as a social signal of submission. Previous studies on salmonid colour change have taken place in tank environments, and often over short periods of time. They have also tended to concentrate on one aspect of behaviour – aggression. Using eye (sclera) colour data collected during a study of foraging behaviour in small groups of juvenile Atlantic salmon (*Salmo salar*) held in a semi-natural environment over a 20-day period, associations between different behaviours and eye (sclera) colour were examined. Status-based differences in sclera colour became obvious three days after the start of the study and persisted for the 20 days. Dominant fish had pale sclera, and this pattern was very stable over the experimental period. In contrast, the sclera colour of subordinate fish (ranks 2-5) fluctuated from day to day. Median sclera colour of fish ranked 4-5 darkened on days they received more aggression, and sclera of rank 2 fish were lighter on days on which they initiated more attacks. Changes in sclera colour of fish ranked 2-4 were more frequent during feeding periods than non-feeding periods. This study confirms that the relationship between eye colour and status described in tanks is also seen in more natural environments, and also that colour change in juvenile salmonids is a complex response to local events.

KEY WORDS: salmon, colour, signalling, foraging, dominance

INTRODUCTION

In a population where there is competition for limited resources, conflict between individuals is a common outcome (Huntingford & Turner 1987). It makes sense to minimise energy wastage and the risk of physical damage by avoiding conflict situations with animals that are better competitors. Modifying your appearance to signal your status or intentions to conspecifics is one way to do this.

Some modifications of appearance may be a product of ageing, or a seasonal change, and are irreversible or last for several weeks or months – the development of red breeding colouration in the 3-spined stickleback (*Gasterosteus aculeatus*) is one such case (Matsuno & Katsuyama 1976). Other modifications may be very rapid and reversible. Rapid, reversible visual signals mediated through differential colour patterns often play an important role in the control of aggressive behaviour in fish (Huntingford & Turner 1987). For example, the colour of the opercular flap and iris become very prominent during aggressive encounters in the pumpkinseed sunfish (*Lepomis gibbosus*). Once dominance has been established, these features remain prominent in the dominant individual, but fade or disappear in the subordinate (Stacey & Chizar 1977).

In salmonids, social subordination results in darkening of the skin and eyes (Keenleyside & Yamamoto 1962; Abbott *et al.* 1985; O'Connor *et al.* 1999; Höglund *et al.* 2000). O'Connor *et al.* (1999) found that the intensity of fights between pairs of newly introduced juvenile Atlantic salmon (*Salmo salar* L.) decreased immediately after the subordinate darkened (within 4 hours of introduction), indicating that darkening may act as a social signal of submission. Several studies have shown that the darkened eye and body colour of subordinate salmonids persists for several weeks following hierarchy formation, and that dominant fish can be readily identified by their lighter colour (Keenleyside & Yamamoto 1962; Abbott *et al.* 1985; R.W. Wilson & S.F. Owen unpublished data).

The studies described above have generally concentrated on colour change in relation to aggression, have taken place in tank environments and have frequently considered only pairs of fish. Also, fish are often held together for short or interrupted periods of time. While conducting a study on the foraging behaviour of Atlantic salmon parr held

in small groups in an artificial stream for 20 days, it became obvious that the relationships between eye colour and behaviour were very complex. Aggression and foraging success appeared to affect eye colour in a manner that varied with individual social status.

We used the data collected in this study to examine the development of differences in eye colour following the introduction of fish into a semi-natural stream environment, and to examine factors affecting eye colour in fish of different social status on a daily basis.

METHODS

Animals, husbandry and experimental conditions

The study fish were non-sibling offspring of wild Atlantic salmon, reared at the SOAEFD Hatchery at Almondbank, Perthshire. The fish were transferred in November 1999 to the University Field Station at Rowardennan, on Loch Lomondside, Scotland, and held in a 1 m^2 tank until needed for experiments. The fish were maintained at the natural photoperiod and were fed commercial salmon food (Trouw) *ad libitum*. All parr were of approximately equal size and were from the upper modal group of the size distribution - individuals that hatched during early 1999 and would smolt and migrate during Spring 2000 - as they have been shown to maintain feeding throughout winter (Metcalfe *et al.* 1988).

The experiment was conducted between January and March 2000, in identical sections of an outside artificial stream fed with a constant exchange of water pumped from Loch Lomond. Each section was 2.4 m long by 60 cm wide by 20 cm deep, bounded by wire mesh screens, lined with gravel, and contained twelve coloured bricks set perpendicular to the current to serve as shelters, current baffles, and visual landmarks (Braithwaite *et al.* 1996). The flume is covered with mesh lids to prevent escape and predation. The exterior flume wall is of opaque fibreglass, while the interior wall is of transparent glass surrounding a covered hide. Water velocity in the centre of the channel was 10-15 cm/s (~ 1 body length/s), a level within the preferred range of 10-12 cm parr during autumn-winter (Rimmer *et al.* 1984, Huntingford *et al.* 1998, Veselov *et al.* 1998).

Fish used in the flume were anaesthetised then weighed, length measured, and individually marked with Alcian blue dye on their fins. Once marked, fish were held separately to recover from anaesthesia, then introduced to the stream sections after nightfall. Observations commenced the next morning (day 1). Five size-matched fish were used in each of the sections, and the experiment lasted 20 days. Data were collected from seven groups of fish.

Fish were fed twice daily on thawed frozen bloodworm washed through plastic tubes that passed from inside the hide, down the side of the flume, and under the fine gravel substrate before emerging at the substrate surface. This method of worm release was designed to simulate natural invertebrate drift off the floor of the stream. Worms were introduced in clumps of 1-5 until approximately 100 worms had been released into the stream during each feed. Each section was flanked (and thus separated) by deeper 'pool sections' that were 1.2 m long, contained fine nets, and trapped unconsumed bloodworm as they passed out of the experimental sections. The screens were cleaned daily after the second feed.

At the end of the 20 days, the fish were killed using terminal anaesthesia and a blow to the head, and sex and growth information were collected.

Behavioural observations

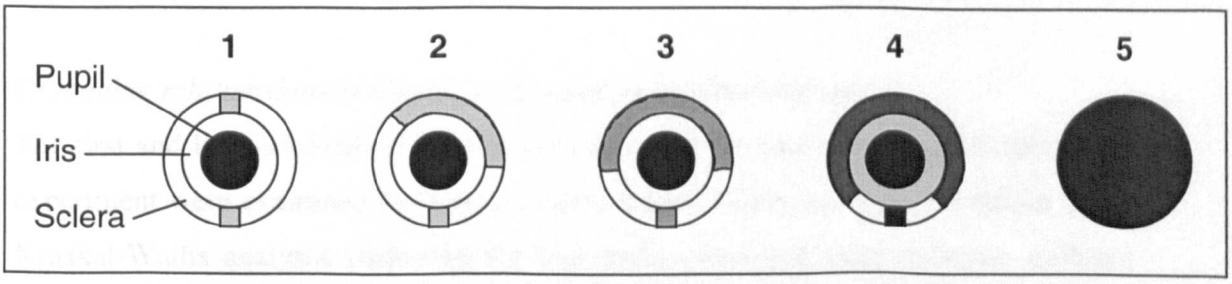
Each group of five fish was observed for approximately 70 minutes a day. These observations were made during both feeding (twice daily, approximately 20 minutes each – times could vary by 1-2 minutes) and non-feeding periods (three times daily, 10 minutes each). During each observation period, the identity of the initiator and recipient was recorded for all aggressive acts. An aggressive act was defined as a charge, chase or bite (Keenleyside & Yamamoto 1962). Where a prolonged chase occurred, each burst of swimming was counted as one act. During feeding observations, a record was also made of which fish took each worm offered.

Scoring eye colour

The eyes of the salmon have a central black pupil surrounded by a narrow, brilliant yellow iris. A broad ring of tissue called the sclera, which can vary from cream to

black (Keenleyside & Yamamoto 1962), surrounds the iris. As the sclera gets darker, the iris fades, to the extreme stage where the entire eye appears black. A pilot study identified five clear patterns of sclera colouration. These patterns and the scores used to define them are shown in Fig. 1. The experiments were conducted during daylight hours under ambient lighting conditions. This meant that lighting could vary between observations, but also within observations as parts of the section could be in shade. To ensure that the sclera scores used were robust under different lighting conditions, the pilot study investigated whether observer perception of sclera score changed as fish moved between sunlight and shade, and whether the same patterns of sclera colour could be observed on overcast and sunny days. From these observations, scoring of sclera patterns appeared to be highly robust and unaffected by ambient lighting conditions.

Figure 1 Patterns of sclera colour and their designated scores. Eyes are drawn as if the fish were facing right.



Before and after each observation period, the group was scanned to identify the sclera score of each individual. Thus, the sclera colour of each individual was scanned ten times each day, giving 6 scores from non-feeding periods, 2 pre-feeding scores and 2 post-feeding scores. Changes in sclera score within an observation period did occur, but were not recorded for logistical reasons.

Assigning social status

Individuals within each group were assigned a social status (ranking from 1: dominant to 5: most subordinate). The number of aggressive acts received and initiated between each pair of fish in a group during the 20 days of observations were totalled. The proportion of interactions won by each fish within each pairing was then calculated, and these proportions organised into an aggression matrix for each group (Martin &

Bateson 1986). The values for each fish were shuffled within the matrix until the proportions were maximised in the bottom left of the matrix. The final order of individuals along the top of the matrix (from left to right) was used to rank the fish (1-5). Throughout this paper, the dominant fish is rank 1, and ranks 2-5 are subordinate.

A variety of methods can be used to rank individuals within groups of juvenile salmonids, including food intake (e.g., McCarthy *et al.* 1992), aggression (Adams *et al.* 1998) and non-aggressive behaviours (e.g., Sloman *et al.* 2000). Aggression appeared to the primary force shaping group dynamics in this experiment, and an aggression-based ranking system was therefore deemed appropriate for use in this study. A comparison of different methods for assigning social status to individuals in these groups will be reported elsewhere.

Two-way ANOVAs of initial weight and length data (using social rank and group as factors) showed that rank was not affected by initial size (weight: $F_{4,24}=1.62$, $p=0.201$; length: $F_{4,24}=1.51$, $p=0.232$).

Examining relationships between social status and colour change

The first and last non-feeding sclera scores recorded for each fish on each day of the experiment were examined for a social status-based difference in sclera colour using Kruskal-Wallis analyses (adjusting for tied ranks). Post-hoc nonparametric multiple comparisons were made using a modified Nemenyi test that considers differences between mean ranks (Miller 1981; Zar 1999).

To examine daily fluctuations in sclera colour, the median basal sclera score (hereafter, basal sclera score) was calculated for each individual for each day. This was defined as the median of the pre-feeding and non-feeding sclera scores recorded on that day. Post-feeding scores were excluded so that any short-term changes in sclera score attributable to the feeding experience would not affect the calculation of a basal sclera score.

The daily proportion of offered worms captured (foraging success) and the level of aggression initiated and received (acts/min) were also calculated for each fish. Basal sclera scores for the twenty days were rank-transformed, and these ranks were then separately regressed against foraging success, aggression initiated and aggression

received for each individual. The regression slopes calculated for each individual were used as single data points in t-tests. This allowed examination of relationships between social status, daily basal sclera score and aggressive and foraging experiences, while avoiding issues of pseudo-replication arising from multiple observations for each individual. A regression slope of zero indicates there is no relationship between sclera score and levels of aggression and foraging success for that fish. Two-tailed t-tests examined whether the average slope was significantly different from zero (regression slope data were normally distributed).

Effects of feeding

Changes in sclera colour over an observation period were examined by comparing the pre- and post-observation sclera scores for each fish and classifying them as the same or different. To examine effects of feeding, these changes were then compared for feeding and non-feeding observation periods. To avoid pseudo-replication, the proportions of changes during feeding and non-feeding periods observed over the 20-day period were calculated for each individual, and the difference between these proportions tested using paired two-tailed t-tests (data were normally distributed).

Ethical note

The experiment was carried out under a U.K. Home Office Licence (#60/2025). Fish were observed several times a day during the course of the experiment, so fish could have been removed if any kind of physical damage had been observed, or if fights had ever escalated to a point where injury could occur. This was never necessary, as overt aggression is rare once dominance hierarchies are established (Metcalfe *et al.* 1989). None of the fish sustained physical damage during the experiments.

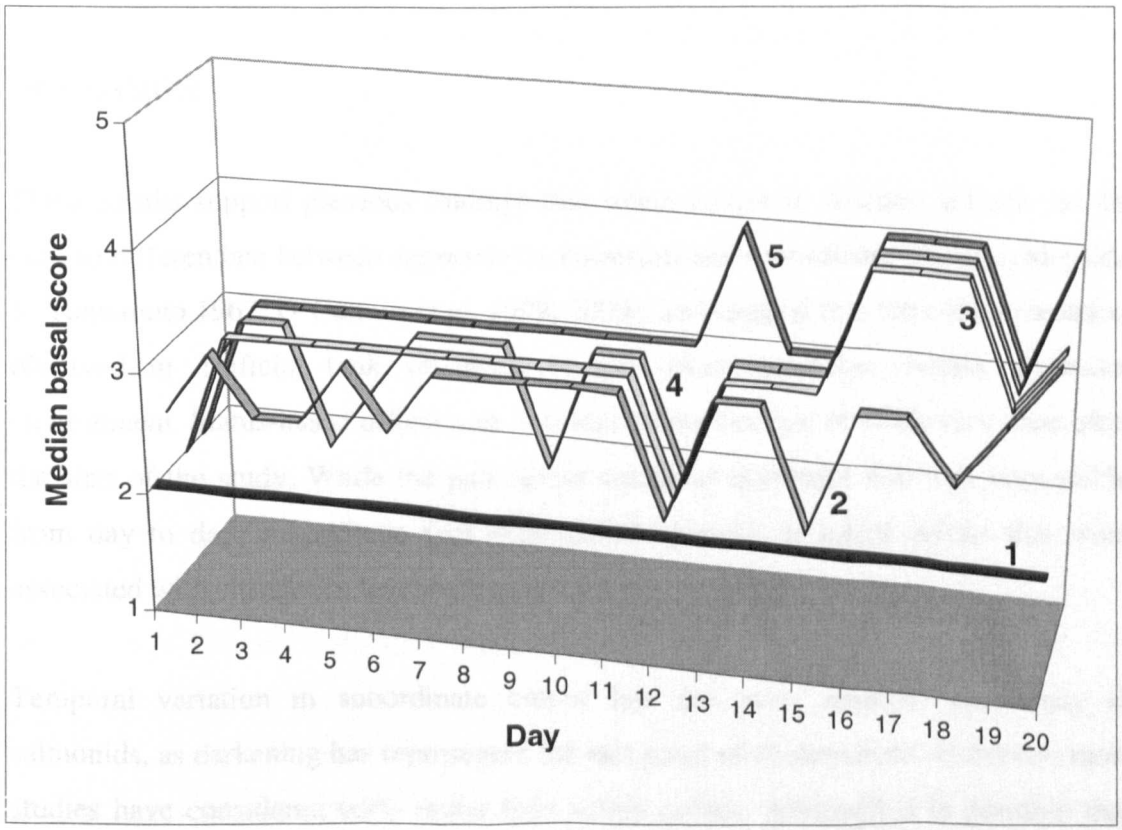
RESULTS

Sclera colour and social status in a semi-natural environment

Figure 2 illustrates how the basal sclera scores of individuals of different rank changed over the course of 20 days. On the first day of the study, there was no significant difference between the initial sclera scores of all fish based on their eventual social status (Kruskal-Wallis test of scores by status: H=1.02, $d.f.$=4, p=0.906). However, by the morning of the third day after the start of the study, there was a significant difference in sclera score between individuals of different ranks (H=15.54, p=0.004),

with the most dominant fish and the most subordinate fish having significantly different sclera scores. These differences persisted to the end of the experiment. On day 20, the final sclera colour of dominant fish was significantly lighter than that of the two most subordinate individuals (fish ranked 4 and 5).

Figure 2 Changes in the median basal sclera score of individuals of different social status over 20 days. Points represent the social rank median of median basal sclera scores of each individual, where the median daily basal sclera score is the median of sclera scores recorded before feeding observations and before and after non-feeding observations. Social status (marked by numbers alongside the appropriate lines) is derived from aggression data collected over the 20 days. The most aggressive (dominant) individual has a rank of 1.



Daily fluctuations in sclera colour: effect of aggression and feeding

The basal sclera score of the dominant individual was very stable, while the basal sclera score of subordinate individuals fluctuated from day to day (Fig. 2). Regression analyses revealed that, on days that the two most subordinately ranked individuals were most heavily attacked, their basal sclera colour was darkest (Two-tailed t-tests of individual regression slopes: rank 4: T=3.54, p<0.05; rank 5: T=3.31, p<0.05). A significant negative relationship was found between basal sclera score and levels of

aggression initiated for rank 2 individuals (two-tailed t-test of regression slopes for rank 2 individuals: T=-2.45, p=0.05), suggesting that on days that these fish initiate more aggression, their basal sclera colour is lighter. All other relationships were non-significant.

The frequency of changes in sclera score during feeding and non-feeding observation periods were compared. The sclera was more likely to change colour during feeding than non-feeding observations (paired t-test: T=4.34, n=35, p<0.001). Repeating the t-tests for each rank separately, this relationship was found to apply only to fish ranked 2 and 3 (T=2.69, p<0.05 and T=3.92, p<0.01 respectively, n=7), although a similar trend was also present in the data for fish ranked 4 (T=2.08, p=0.082).

DISCUSSION

These results support previous findings that sclera colour in Atlantic salmon can be used to differentiate between aggressively dominant and subordinate fish (Keenleyside & Yamamoto 1962; O'Connor *et al.* 1999, 2000), and suggest that the colour variation observed in artificial tank situations is also likely to occur within a stream environment. Status-based differences in sclera colour became obvious very soon after the start of the study. While the pale sclera colour of dominant fish was very stable from day to day, subordinate fish experienced changes in sclera colour that were associated with changes in levels of aggression and foraging activity.

Temporal variation in subordinate colour has not been reported previously in salmonids, as darkening has represented the end point of observations. However, most studies have considered body rather than sclera colour. Although it is possible that fluctuations in eye colour are not always matched by corresponding shifts in body colour, O'Connor *et al.* (1999) reported a highly significant association between body and sclera colour of juvenile Atlantic salmon. Body colour was not scored during this experiment because of time constraints during observations, but change in body and sclera colour did appear to be positively related. The darkest sclera score (when the eye is completely black) seemed to be associated with very dark body colour (see O'Connor *et al.* (1999) for descriptions of body colouration).

Looking at the social ranks separately, various relationships emerged between sclera colour and levels of aggression. Levels of aggression within each group varied from day to day, probably as a result of changes in individual motivational status. All subordinates had pale sclera at the start of the study, but subsequently darkened. The ranks were aggression-based, with subordinates receiving proportionally more attacks by definition. Thus, the strongest relationship that could be expected to emerge from the data would be that between levels of aggression received and darkening, particularly within the most subordinate ranks. This was found to be the case; the sclera of the two most subordinate ranks of fish (ranks 4 & 5) being darker on days they were attacked more. The sclera of rank 2 fish were lighter-coloured on days on which they initiated more attacks. These findings again illustrate that the sclera of subordinate fish did not simply darken and remain dark, but that they were responsive to daily variation in the aggressive environment. The above associations, detected on a daily basis, could be considered a conservative estimate of the associations occurring on a finer time-scale.

Status-based differences in the relationship between sclera colour and feeding activity were also apparent. The sclera colour of intermediate-ranked fish (ranks 2-4) was more likely to change during feeding than non-feeding observations, while the sclera of the most dominant and most subordinate individuals were unaffected by foraging activity. So sclera changes occurred during feeding in intermediate-ranked fish, but not in fish at the extreme ends of the social scale. Dominant sclera colour appears to remain light regardless of foraging experience, while the sclera colour of the most subordinate fish appears to remain dark.

Subordinate activity results in an increased chance of social interaction, which may lead to aggression. It makes sense to minimise interaction with the dominant (or higher ranked subordinates) during non-feeding periods, when there is little or no benefit to be had by fighting. During feeding, subordinate fish may be more active and thus involved in more social interactions that might influence sclera score. *Ad hoc* observations during the study showed that successful foraging was associated with sclera change, so changes recorded during feeding periods may not be solely due to increases in aggressive interaction.

Sclera colour was observed to change very rapidly (frequently within a minute) and often more than once during an observation period. On several occasions, subordinate fish were observed successfully feeding, and lightening, then being attacked and darkening within one observation period. Subordinate foraging activity is known to elicit attacks from dominant fish (Newman 1956, Jenkins 1969), but sclera lightening may also attract the attention of the dominant. Abbott *et al.* (1985) found that subordinate steelhead trout (*Oncorhynchus mykiss*) maintained their dark body colouration when fed in isolated from the dominant individual. It may be that successful foraging in full view and attack range of the dominant triggers a neural response that results in lightening of the subordinate sclera, a response that is absent if subordinates are fed in visual isolation. Clearly, experiments designed with the sole purpose of examining colour change during feeding need to be conducted. The very rapid change in sclera colour observed during successful foraging is likely to be under neural control, while the darker, basal sclera state of subordinates is more likely to be under the control of various neural and pituitary hormones (Fujii & Oshima 1986; Höglund *et al.* 2000). Elucidating the mechanism by which foraging success results in lightened sclera could provide an interesting comparison with mechanisms involved in translating aggressive stimuli into colour changes (Höglund *et al.* 2000).

Groups of juvenile salmon within streams form very stable social structures. While some fish are mobile, most maintain the same small home ranges for prolonged periods (Kalleberg 1958). Some fish aggressively defend a territory based around a profitable feeding site, while others 'float' between the territories of more dominant individuals (Kalleberg 1958; Puckett & Dill 1985). Interactions between floating and territorial individuals are likely to be frequent during foraging, and the ability to communicate social status during these interactions could help to prevent unnecessary and potentially costly conflict (O'Connor *et al.* 1999). However, status signalling through body colour may be disadvantageous in a stream situation, as pale individuals could be more visible to predators than their darkened, cryptic, conspecifics. Ignoring predation risk and signalling hypotheses, cryptic colouration in a natural situation may simply help subordinates to escape the attention of territory holders.

The results discussed here have demonstrated that subordinate juvenile Atlantic salmon have darker sclera than aggressively dominant individuals under semi-natural conditions. Also, under these conditions, short-term behavioural experience can affect

sclera colouration, especially in subordinate fish. Sclera darkened when subordinates were attacked more frequently, but were often observed to lighten in relation to increased foraging success. Foraging, and possible lightening, of the most subordinate fish elicited attacks from the dominant that resulted in sclera darkening. Fish of rank 2 lightened on days they initiated more aggression. The results suggest that subordinate rank and the aggressive response of the dominant affect the stability of sclera colour. Future work on the stimuli and mechanisms controlling colour change in salmonids under natural or semi-natural conditions would contribute to a better understanding of subordinate survival strategies in populations of juvenile salmonids.

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Appendix 5 Transfer of cortisol in and out of unfertilised eggs

Background

Chapter 4 examined intra- and inter-nest variation in egg steroid content of brown trout eggs. I hypothesised that egg steroid content could change between ovulation and spawning, and that full-sibling eggs spawned at different times could therefore have different steroid concentrations. To add support to this theory, we investigated the movement of cortisol between ovarian fluid and ovulated eggs. Here I describe the results of an experiment where I exposed ovulated Atlantic salmon eggs to ovarian fluid in which cortisol concentrations were artificially elevated. By transferring eggs back into unmanipulated ovarian fluid, I was able to assess movement of cortisol out of the eggs also.

Methods

Eggs and ovarian fluid were collected from a ripe Atlantic salmon stripped at the Fisheries Research Services Freshwater Laboratory at Almondbank, Perthshire. Fifty eggs were immediately blotted dried, pooled in batches of 3 in Eppendorf tubes, and these eggs and a sample of ovarian fluid were then frozen for later hormone analyses (to determine initial levels of cortisol).

A further 5 ml of ovarian fluid was added to a 20 ml glass vial, in the base of which 100 μg of cortisol (in 100 % A.R. ethanol) had been evaporated. After thorough vortex mixing, 30 eggs were counted into the vial, and the container held at 4 °C (equivalent to the water temperature). At 2, 4, 8, 16 and 24 hours after the eggs were placed in the vial, 1 ml of fluid was sampled and frozen, and 6 eggs were removed and rinsed in spare ovarian fluid. Three of these eggs were then pooled and frozen for hormone analyses, while the remaining three eggs were transferred to an eppendorf containing 0.5 ml of unmanipulated ovarian fluid and held at 4 °C. After a period of time equal to that spent in the cortisol-treated fluid, the eggs and fluid were removed from the eppendorfs and separately frozen for hormone analyses. At 16 h, treated eggs were accidentally frozen rather than transferred to untreated ovarian fluid.

The amount of cortisol used was such that a concentration gradient should have been established, facilitating the movement of cortisol into the eggs (steroids are lipophilic and can cross membranes, Feist *et al.* 1990). Assuming the eggs had taken up cortisol during the first phase, transferring the eggs into unmanipulated fluid would reverse the cortisol gradient between fluid and egg, resulting in the movement of cortisol from eggs to fluid.

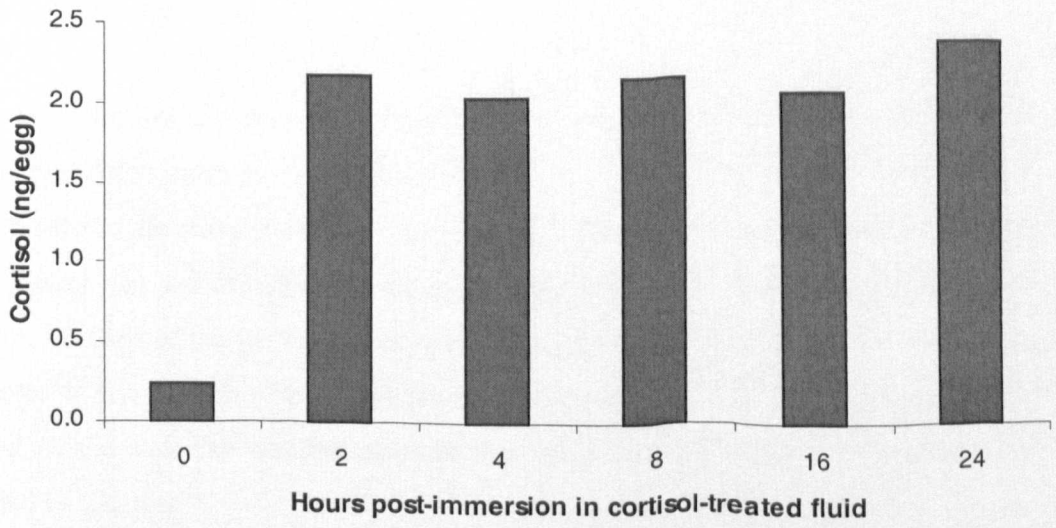
Samples were held at 4 °C and gently agitated throughout the course of the experiment (48 h). At all stages the eggs were kept well removed from water, as this would have caused 'waterhardening,' the absorption of water across the egg membrane and a subsequent hardening of the egg membrane.

Hormones were extracted from eggs and fluid as described in Appendix 1.

Results

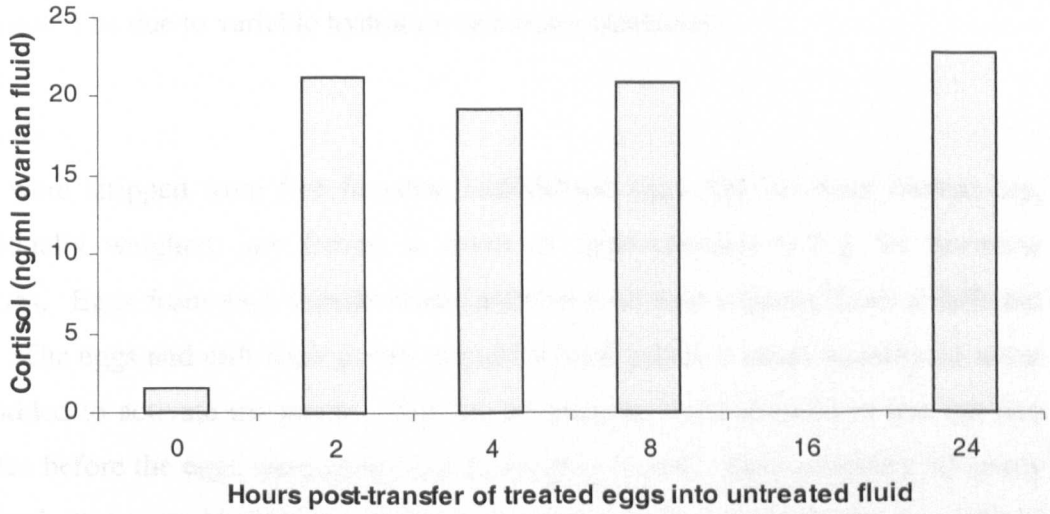
There is an unfortunate lack of replication in this trial. However, it appears that cortisol can move from ovarian fluid into ovulated eggs and that this movement occurs fairly rapidly (Figure 1). The lack of further increases after 2 hours in the treated fluid suggests that the eggs may have reached some sort of concentration equilibrium with the fluid surrounding them.

Figure 1 Egg cortisol content after immersion in cortisol-treated ovarian fluid (n=1 at each time point).



Similarly, cortisol appears to be able to move out of the treated eggs and into untreated fluid (Figure 2). Again, some sort of equilibrium situation appears to be reached rather rapidly. It is possible that some of the increase in fluid cortisol concentration (and in the egg measurements above) could be due to cortisol adhering to the surface of the egg during transfer from treated to untreated fluid (or transfer to clean Eppendorf tubes for freezing prior to homogenisation). Every effort was made to rinse the eggs during transfer stages to try to minimise this error. The results of Chapter 4 give support to the results of this trial, however, as there were significant changes in steroid content of eggs spawned in different nests by the same females.

Figure 2 Transfer of cortisol from cortisol-treated eggs to untreated ovarian fluid (n=1 at each time point).



Appendix 6 The hydration and clearance of hormones from eggs within a redd during the 24 hours post-fertilisation

Background

Chapter 4 examined inter and intra-nest variation in the size and hormone content of brown trout eggs. The study animals used in that chapter were mature wild adult non-migratory brown trout captured from a hill loch near Pitlochry, Perthshire, Scotland during November 2000. In Chapter 4, eggs were excavated from artificial spawning sections at 4 hours post-spawning, as I believed that 4 hours would allow for complete hydration of the eggs, thus permitting a 'standardised' comparison between eggs from different nests. Here I examined how egg weight and cortisol content changed between spawning and sampling using eggs from a further five pairs of fish, to properly establish whether inter-nest variation in egg size or hormone content after 4 hours could be due to variable hydration and water hardening.

Methods

Eggs were stripped from five females. Unfertilised eggs (30-50) were blotted dry, individually weighed, and frozen in pools of approximately 0.2 g for hormone analyses. Eggs from each female were fertilised with milt stripped from a different male. The eggs and milt were gently mixed by hand before a small quantity of water was added to activate the sperm. The mixed gametes were allowed to rest for five minutes before the eggs were gently but thoroughly rinsed. Approximately 50 newly fertilised eggs were blotted dry, individually weighed, and again frozen in pools of approximately 0.2 g.

Further sub-samples of approximately 50 newly-fertilised eggs were buried in each of seven gravel-filled mesh cylinder 'nests' (diameter 15 cm), which were then placed in the excavated redds (long mounds of gravel) created by the spawning pairs in Chapter 4. The gravel was 1-8 cm in diameter, and the eggs were effectively buried 8-10 cm below the surface of the gravel – equivalent to the location of naturally spawned eggs in Chapter 4. The surface of the gravel in the nests was level with the gravel surface in the redd. Water velocity was the same as that used during the spawning experiment described in Chapter 4.

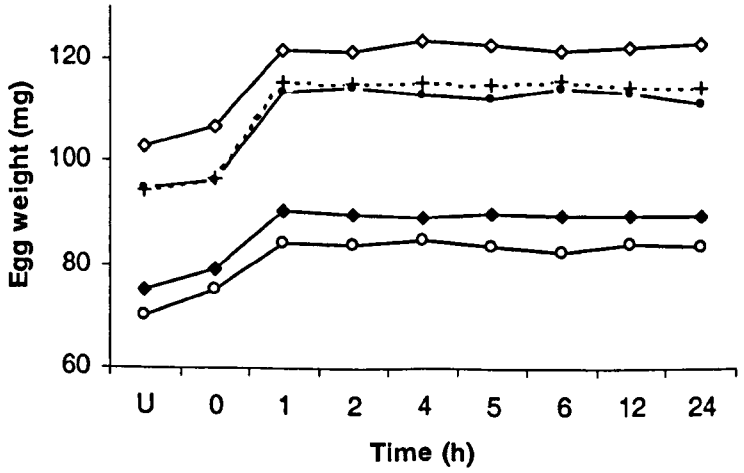
Nests from each pair were recovered 1, 2, 4, 5, 6, 12, and 24 hours after being placed in the redds. The nests were emptied onto a sieve (1 cm mesh size) over a water-filled container. The eggs passed through into the container, and, using forceps, were immediately transferred into petri dishes lined with moist tissue until they could be blotted, weighed and pooled as described above. Any eggs which had white spots or had turned completely opaque (indicative of egg inviability) were removed from the sample. All eggs were frozen within 30 minutes of excavation.

Cortisol levels were measured in eggs of one female only because of financial and time constraints. Hormone extraction and measurement were as described in Chapter 4.

Results

Figure 1 illustrates that eggs from all 5 females underwent hydration following fertilisation and immersion in the redd, but that hydration had effectively ceased by 1 hour post-immersion. Mean % hydration ± 1 s.e.m. at 1 hour post-immersion (relative to unfertilised egg weight) was 20.2 ± 0.7 %. Standard errors for each sampling point were very small (< ± 1 mg) and are therefore not shown on the graph.

Figure 1 Changes in egg weight following fertilisation and immersion in redds. U=unfertilised, 0=post sperm-activation.

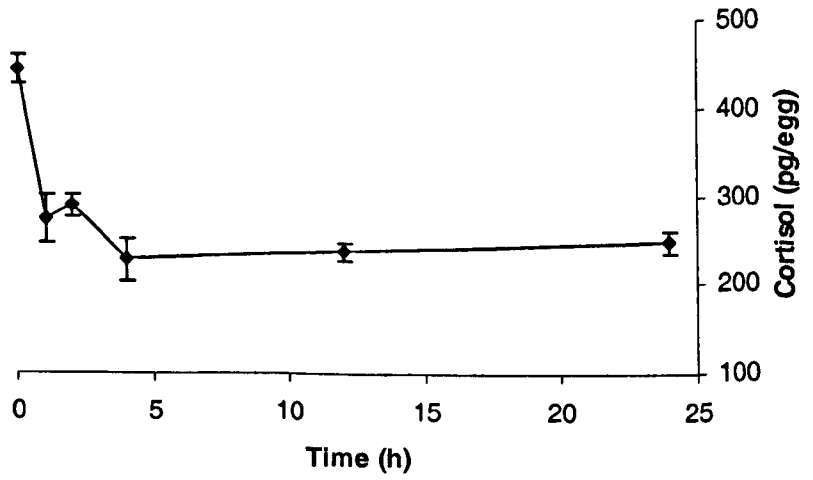


There were also changes in egg cortisol content after fertilisation, although cortisol content in the eggs from the other four females should be measured before the trend shown in Figure 2 can be confidently said to be the general pattern. There was a 38 % reduction in cortisol content of eggs between fertilisation and 1 hour post-immersion. By the 4-hour sample, a further 10% of the original cortisol had been lost, but cortisol content did not change between the 4 and 24-hour samples.

Conclusion

It appears that the 4-hour delay between spawning and sampling used in Chapter 4 ensured that eggs from all nests were completely hydrated and water hardened at sampling. Post-spawning increases in egg weight and losses of egg cortisol had stabilised by this time. It is possible that changes in the content of other hormones may not parallel that of cortisol. However, one would expect a similar pattern for all steroid hormones, including testosterone and 17α,20β-dihydroxy-4-pregnen-3-one, the other steroids examined in Chapter 4. We therefore conclude that the egg data collected in Chapter 4 are suitable for inter-nest comparisons, and that any variation found in that chapter is real, and not a product of the sampling protocol employed.

Figure 2 Changes in egg cortisol content following fertilisation and immersion in the redd. Values are means ± 1 standard error.



Appendix 7 Broodstock and spawning details from Chapter 4

Pair	Date start	Date end	Nest #	Date	Spawn time	Night/Day	Excavation time	Eggs frozen	Eggs reared	Total # eggs
1	14/11/00	20/11/00	1	15/11/00	1100	d	1500	51	167	226
1	14/11/00	20/11/00	2	17/11/00	0330	n	0730	23	35	59
1	14/11/00	20/11/00	3	18/11/00	0830	d	1230	0	10	10
2	14/11/00	17/11/00	1	15/11/00	0950	d	1350	63	261	324
2	14/11/00	17/11/00	2	15/11/00	2320	n	0330	26	50	78
2	14/11/00	17/11/00	3	16/11/00	1555	d	1955	24	10	35
3	14/11/00	17/11/00	1	15/11/00	1200	d	1600	51	150	204
3	14/11/00	17/11/00	2	15/11/00	2320	n	0245	45	96	147
3	14/11/00	17/11/00	3	16/11/00	0905	d	1305	21	44	69
4	20/11/00	23/11/00	1	20/11/00	2310	n	0320	40	184	224
4	20/11/00	23/11/00	2	21/11/00	0950	d	1400	40	91	131
4	20/11/00	23/11/00	3	21/11/00	1910	n	2320	20	29	49
4	20/11/00	23/11/00	4	22/11/00	0415	n	0915	12	14	26
5	20/11/00	23/11/00	1	20/11/00	2215	n	0215	34	213	247
5	20/11/00	23/11/00	2	21/11/00	0900	d	1250	40	192	232
5	20/11/00	23/11/00	3	21/11/00	1730	d	2130	20	102	122
5	20/11/00	23/11/00	4	22/11/00	0415	n	0830	24	40	64
6	20/11/00	23/11/00	1	20/11/00	2310	n	0405	40	122	162
6	20/11/00	23/11/00	2	21/11/00	1055	d	1545	40	122	162
6	20/11/00	23/11/00	3	21/11/00	1910	n	0005	40	98	138
6	20/11/00	23/11/00	4	22/11/00	0415	n	0940	20	65	85
6	20/11/00	23/11/00	5	22/11/00	1230	d	1705	12	25	37
7	20/11/00	23/11/00	1	20/11/00	2310	n	0440	48	115	163
7	20/11/00	23/11/00	2	21/11/00	0900	d	1430	42	106	148
7	20/11/00	23/11/00	3	21/11/00	1910	n	0055	-	48	48
7	20/11/00	23/11/00	4	22/11/00	0415	n	1000	21	57	78
7	20/11/00	23/11/00	5	22-23/11/00	2200-0600	n	1320	-	46	46

	Female			Male		
Pair	Initial wt	Final wt	FL	Final wt	FL	Eggs left
1	243	201	29.0	225	29.2	3
2	303	248	30.8	296	31.2	1
3	181	148	25.5	189	26.5	0
4	326	266	32.1	347	33.0	0
5	370	284	33.1	354	32.8	3
6	357	284	32.3	354	33.1	0
7	285	236	29.6	262	29.5	0

Appendix 8 Alkali-labile Phosphate Assay Reagents

The alkali-labile phosphate (ALP) assay was used to determine the concentration of ALP in plasma of maturing female brown trout (Chapter 7). ALP is a good estimate of plasma vitellogenin, the major constituent of the egg. Phosphate was extracted from plasma following the method of Wallace & Jared (1968), and quantified using the method of Stephens (1963). The protocol is detailed in Chapter 7. Here, I provide details of the reagents used in the quantification of ALP.

Phosphate Standard Solutions

- 0.2197 g of Potassium dihydrogen orthophosphate (KH2PPO4) in 1000 mL flask (50 $mg.L^{-1}$ solution, stock A).
- 20 mL of stock A diluted to 1000 mL to obtain 1000 $\mu g.L^{-1}$ (stock B).
- Aliquots of 10, 20, 30, 40, 50, 60 & 70 mL diluted to 100 mL in calibration flasks.

L-Ascorbic Acid Solution

Dissolve 5.4 g of L-ascorbic acid in 100 mL distilled water. To be made up every 14 days as solution oxidises with time.

Sodium Molybdate Solution

Dissolve 4.11 g of sodium molybdate ($Na_2MoO_4.2H_2O$) in 100 mL distilled water.

5N Sulphuric Acid

136 mL conc. sulphuric made up to 1000 mL.

Antimony Potassium Oxide (+)Tartrate 0.5-hydrate solution

Dissolve 0.342 g in 100 mL distilled water.

Trichloroacetic acid solution

Dissolve 10 g (10%) and 5 g (5%) in 100 mL distilled water.

MIXED REAGENT

Freshly prepared each time and mixed after each addition.
5 parts 5N sulphuric acid
2 parts sodium molybdate
2 parts L-ascorbic acid
1 part antimony potassium oxide tartrate