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Evaluation of biochemical methods for the non-destructive identification of sex in upstream migrating salmon and sea trout

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This study was designed to evaluate methods for non-destructively identifying the sex of upstream-migrating adult salmon or sea trout intercepted at a point in the reproductive cycle where external secondary sexual characteristics are not sufficiently informative to assist with identification of sex. Female-specific markers of reproductive activity (plasma 17β-estradiol, E2; vitellogenin, VTG; alkali-labile phosphoprotein phosphorous, ALP) were measured over 12 months in a captive population of brown trout. During the early months of the reproductive season (February – May) and using the concentration of plasma E2 or plasma ALP as a marker for females the proportion of fish in which sex was misidentified was high (15% - 50%). The misidentification rate was considerably lower (1 – 8%) using plasma VTG. Preliminary evaluation of a commercial immunochromatographic VTG test system as a screen for the presence or absence of VTG in plasma from brown trout provided results that were consistent with those obtained from direct measurement of plasma VTG levels by ELISA. These preliminary conclusions were verified by sampling upstream-migrating sea trout and salmon trapped over a 6 month period. Plasma E2 levels did not satisfactorily discriminate between male and female sea trout and salmon. However, plasma VTG levels in both species were bimodally distributed and it was assumed that this divergence corresponded to male (plasma VTG levels <10 μg ml^{-1}) and female (plasma VTG levels >800 μg ml^{-1}) fish. Plasma ALP provided a more accurate indication of sex in the wild salmon and sea trout than was suggested by the pilot study on captive brown trout. The commercial immunochromatographic VTG test system provided results that were wholly consistent with the data obtained from the trapped fish by direct measurement of plasma VTG.

Key words: upstream migration; *Salmo salar*; *Salmo trutta*; 17β-estradiol; vitellogenin; alkali-labile phosphoprotein phosphorous.

INTRODUCTION

The efficient management of Atlantic salmon (*Salmo salar* L.) and sea trout (*Salmo trutta* L.) stocks requires that recruitment is optimised and this requirement is addressed by setting targets for critical phases of the recruitment process. A key target is that set for spawning (Milner *et al*., 2000) which requires that the spawning biomass is estimated each year. However, a major difficulty encountered in accurately estimating the spawning biomass is in obtaining river-specific data for the sex ratio of returning adult fish. Because of the timing of the seawater to freshwater migration in many salmonid populations secondary sexual characteristics have yet to become expressed sufficiently to allow the external identification of sex in fish that are intercepted during the upstream migration. This problem has been highlighted in two reports (Solomon 1995; Harris 2001), both of which recommended the development of alternative methods for the identification of sex.

Non-destructive identification of the sex of an adult salmon or sea trout in which no clear secondary sexual characteristics (e.g. a kype in male fish) are evident can be addressed in two ways. The first is to utilise a probe for a sex specific marker at the level of the genotype. This approach requires the identification of sex-linked genetic markers, comprising sequences of DNA that are unique to one sex or the other (Devlin and Nagahama 2002). Genetic sex markers have been characterized for several fish species (*Oncorhynchus tshawytscha* Walbaum; Clifton and Rodriguez 1997, Devlin *et al*. 2001; *Clarias gariepinus* Burchell; Kovacs *et al*. 2000; *Gasterosteus aculeatus* L.; Griffiths *et al*. 2000) but difficulties have been reported in identifying a similar marker for use in Atlantic salmon (*Salmo salar* L; McGowan and Davidson 1998) and at this time there is no specific genetic marker of sex available for *Salmo* species.

The second approach is to utilise sex-specific phenotypic alterations associated with reproductive development in order to identify sex in early reproductive phase fish. Sex-specific changes in the endocrine system of fish, associated with reproductive development, are detectable 12 months or more prior to spawning. The most pronounced of these is the estrogen-dependent elevation of the yolk protein precursor vitellogenin (VTG) in the blood of female fish (Copeland *et al*, 1986).

The implementation of a biochemical marker of sex as an operational tool requires consideration of its ease of use outside the research laboratory environment. Both E2 and VTG are routinely quantified by radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) but both of these methods require specialised equipment, and the species specificity of VTG assays limits the availability of reagents. However, because for this purpose the precise quantification of VTG concentrations is not required, surrogate measures of VTG can be exploited. The VTG molecule contains a high proportion of calcium and phosphate and consequently plasma levels of both calcium and protein-associated phosphorous have been utilised as indirect markers of VTG concentration (Kramer *et al*., 1998; Gillespie & de Peyster, 2004). Alkali-labile phosphoprotein phosphorous (ALP) is considered to provide a more accurate indicator of VTG concentration than calcium because the former is measured specifically in the serum fraction containing phosphorylated lipoproteins, of which VTG is the quantitatively dominant component in mature female salmonids (Nagler *et al*., 1987). The measurement of ALP can be accomplished using readily available reagents and requires equipment found in most analytical chemistry laboratories. The recent introduction of an immunochromatographic test kit offers a more crude detection level (present or absent) for VTG but greater specificity than the ALP assay. This kit is

designed for the detection of VTG in masu salmon (*Oncorhynchus masou* Brevoort) at a presence/absence level of reporting but is suitable for use in other species of salmonids including *Salmo* spp. (for details, see Materials and Methods). The measurement of E2, VTG, or ALP requires that a blood sample be obtained from the fish. This can be achieved safely and humanely in sedated fish. However, there are reports in the literature suggesting that VTG is present, and can be detected, in the superficial mucus of maturing female fish (Chang *et al*., 1996) potentially obviating the need for blood sampling.

The aim of this study was to contribute to the provision of a practical management tool for the identification of the sex of upstream migrating salmonid fish by: (i) in a laboratory study, evaluating the relative accuracy and sensitivity of plasma ALP, E2, and VTG, and mucosal VTG, as markers of sex at different stages in the reproductive cycle of brown trout, and; (ii) in a field trial, assessing the utility of the selected indices in non-destructively identifying the sex of salmon and sea trout intercepted during upstream migration.

MATERIALS AND METHODS

COMPARISON OF MARKERS OF SEX IN CAPTIVE BROWN TROUT.

Fish and holding conditions

In February 2001, 500 two-year-old brown trout (*Salmo trutta*; Dunsop Bridge Trout Farm, Clitheroe) were distributed equally between five outdoor tanks (1.8 m diameter, 1000 l, constant flow-through of lake water 30 l min^{-1}). The fish were fed once daily (Skretting Standard Expanded trout feed), five days $week^{-1}$, at approximately 1% body weight day^{-1}. The

fish experienced a natural photoperiod and water temperature profile (range: 3.6ºC in March - 17.9ºC in August).

Sampling protocol

The experimental population was sampled at two-weekly intervals from mid-February until November with one final sample in January 2002 (19 samples). Each sample comprised 20 fish, four from each tank. Fish were rapidly netted into anaesthetic (2-phenoxyethanol, 1:2000) and immediately after sedation, mucus was collected from the first fish into a glass dish by holding the fish vertically and scraping the mucus downwards using a large stainless steel spatula. The collected mucus (1.5 – 3.0 ml) was transferred to a capped polypropylene tube and stored frozen (-70ºC) until required for assay. A blood sample was taken from the caudal vessels into a heparinized syringe and each fish was killed by a blow to the head. Fish were weighed, measured, and gonads and liver were removed and weighed. Sex was ascribed to each fish on the basis of visual inspection of the gonads. Blood was stored on ice (< 1h) until being centrifuged to separate plasma. Plasma was pipetted into three capped 1.5 ml polypropylene tubes and stored frozen (-70ºC) until required for assay. Plasma samples were analysed for estradiol-17β (E2), vitellogenin (VTG), and alkali-labile phosphoprotein phosphorous (ALP). These measurements were later used to assess which indicator was most effective at discriminating between male and female fish, and how early this discrimination could be applied. Plasma testosterone (T) was also measured in both sexes to confirm that the reproductive cycle proceeded normally in both male and female fish in the captive population.

COMPARISON OF MARKERS OF SEX IN UPSTREAM MIGRATING SEA TROUT AND SALMON

Sampling protocol

Upstream migrating salmon and sea trout were sampled at the Environment Agency (EA) Forge fish trap on the River Lune at Halton (Lancashire, UK) on 17 occasions between April and October 2002. Blood samples were collected while the fish were sedated (2-phenoxyethanol; 1:2000) for weighing and measuring. In order to minimise damage to the fish and to limit residual blood loss, blood was removed from the Cuverian ducts into heparinized syringes. After transfer to capped polypropylene tubes the blood was kept on ice in an insulated container until returned to the lab (<2h) where it was immediately centrifuged and plasma was aspirated and stored frozen in aliquots for subsequent analysis. Plasma samples were analysed for E2 and VTG. The trapped fish were released after weighing, measuring, scale removal, and blood sampling.. Therefore no definitive identification of sex, based on inspection of the gonads, was available. Instead, sex was attributed initially on the presence of secondary sexual characteristics at the time of capture by Environment Agency staff operating the trap, and subsequently on the basis of plasma VTG levels, as described below

ASSAY METHODS

Plasma levels of T and E2 were determined in ethyl acetate extracts of plasma using previously validated radioimmunoassays (Pottinger & Pickering, 1985; Pottinger & Pickering, 1990). Plasma ALP was extracted from plasma using the method of Wallace and Jared (1968) and was estimated as inorganic phosphorous using a colorimetric method based on that of Stephens (1963). Plasma VTG levels were measured in plasma using an established ELISA

method (Kirby *et al*., 2004). Preparation and purification of Atlantic salmon VTG was closely based on the method described by Norberg (1995) for the preparation of Atlantic halibut *Hippoglossus hippoglossus* L. VTG. The antiserum, raised against *Salmo trutta* VTG, was a gift from Dr Birgitta Norberg (Norberg & Haux, 1988) and cross-reacts with VTG from both *Salmo* species. The presence or absence of VTG in plasma samples was also evaluated using an immunochromatographic test kit ("Best Checker", Science Tanaka Co. Ltd, 777-13 Shinkoh Nishi 1-chome, Ishikari, 061-3241 Japan) which was designed for the detection of VTG in blood samples from salmonid fish. The antibodies employed in the test system were raised against masu salmon (*Oncorhynchus masou*) VTG but cross-reactivity with Atlantic salmon VTG had been confirmed (Pers. comm. Professor Toshiaki Fujita, Hokkaido University). Plasma samples from four male and four female fish were selected from each of four sample points and were applied to test sticks in 100µl aliquots at dilutions of 1:10 and 1:100 (diluted with phosphate buffered saline: 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4; Sigma). Positive results were denoted by the appearance of ablue line within the test window. The presence or absence and intensity of the line were scored 0 – 3 for each sample.

STATISTICAL ANALYSIS

Analysis of seasonal changes in plasma steroid, ALP and VTG levels in captive male and female brown trout was carried out by ANOVA (Genstat). The following procedure was adopted in order to evaluate the relative effectiveness of each indicator in identifying the sex of individual fish. The assumption was made that females would be characterised by having higher levels of E2, ALP or VTG than males. Therefore, a cut-off point was established for each endpoint, values above which indicated that the fish was female and below which it

would be identified as male. For the captive brown trout population it was possible to determine a cut-off point for each sample time, rather than a single cut-off point for the time-course in its entirety. This provided greater resolution than would be possible if a single cut-off point were employed and took into account the fact that seasonal fluctuations occur in gonadal steroid levels and VTG levels over and above those directly due to sexual maturation. For each indicator (E2, ALP, VTG), at each time point (1 – 19) the mean and standard deviation for each data set were calculated and normal distributions were derived (Microsoft Excel function NORMDIST). Subtracting the frequency values obtained for each interval of the distribution for male fish from those calculated for female fish identified the point at which the distributions converged (i.e. [endpoint] where freq male – freq female = 0). The area under the male distribution to the right of the crossover was considered to represent the proportion of males that could not reliably be distinguished from females if the crossover were used to sort the data. The area under the female curve to the left of the crossover represented the proportion of females that could not reliably be distinguished from males.

RESULTS

INDICATORS OF SEX IN CAPTIVE BROWN TROUT

Somatic data

A total of 380 fish were sampled from the captive population with an overall sex ratio (male:female) of 1:1.7. The mean individual body weight of the test population increased from 391 ± 15 g (mean ± SEM, n = 20) in February 2001 to a maximum of 681 ± 33 g in November 2001. Fork length increased from 31.6 ± 0.4 cm to 37.9 ± 0.5 cm during the same

period.

Gonadal steroids

Mean plasma E2 and T levels showed significant variation during the study period. There was no significant difference in mean plasma T levels between male and female fish during the periods February – May and June – August (<5.0 ng ml^{-1}). From mid-August onwards there was a marked increase in T levels in both sexes, reaching maximum levels in male fish of 18.7 ± 1.4 ng ml^{-1} in October and in female fish of 30.3 ± 2.6 ng ml^{-1} during November. Plasma E2 levels in female fish increased slowly from 0.6 ± 0.1 ng ml^{-1} in February to 2.3 ± 0.4 ng ml^{-1} in September and rapidly rose to 11.1 ± 1.3 ng ml^{-1} in October, subsequently declining to 2.4 ± 0.9 ng ml^{-1} by the end of November. With the exception of two samples (March 01, February 02) mean plasma E2 levels in male fish did not exceed 0.25 ng ml^{-1} and levels in female fish were significantly (P<0.001) higher throughout.

Plasma ALP and VTG

No differences in plasma ALP levels between male and female fish were evident until late April (Figure 1a). From this point onwards, female plasma ALP levels were significantly higher (P<0.001) than those in male fish, rising from 6.7 ± 1.0 μg ml^{-1} to reach a maximum of 89.3 ± 8.4 μg ml^{-1} in November. In male fish, throughout the study period, plasma ALP levels did not exceed 5.0 ± 0.4 μg ml^{-1}. Mean plasma VTG levels were significantly higher (P<0.001) in female fish than in males at every time point (Figure 1b). Mean levels in male fish ranged between undetectable and 48 μg ml^{-1}. In female fish mean levels increased from 342 ± 107 μg ml^{-1} in February to 33730 ± 8827 μg ml^{-1} in October. There was a clear

relationship between individual ALP and VTG measurements, a regression of ALP on VTG gave an r^2 value of 0.93 (y = 0.0016x + 4.5774). It was not found possible to detect ALP or VTG in superficial mucus collected from brown trout, even those in which plasma VTG levels were elevated.

Evaluation of the "Best Checker" immunochromatographic vitellogenin detection kit

At a dilution of 1:100 15 male fish gave negative results and 1 gave a positive result despite having very low VTG levels. Of the 16 females tested, 14 gave positive results and 2 gave negative results. In the ELISA, the latter presented with very low VTG levels (118 μg ml^{-1} and not detectable, respectively). At a dilution of 1:10 the results were not further improved; 3 females with high VTG levels provided a negative result while positive results were obtained with 11 fish. The results obtained with 1:100 diluted plasma were considered to be sufficiently encouraging to further evaluate the test stick system with plasma samples from the trapped salmon and sea trout.

Statistical evaluation of the effectiveness of each indicator in discriminating between sexes

Figure 2 depicts distributions derived from ALP data obtained from the fish sampled on 29.5.01 (males 2.2 ± 0.3 μg ml^{-1}, n = 7; females 8.5 ± 0.9 μg ml^{-1}, n = 13). The crossover point is located at 3.98 μg ml^{-1} ALP. The area under the male distribution to the right of the crossover is 0.0156 while that under the female distribution to the left of the crossover is 0.0807. Therefore, in this case, in any random sample of fish from this population, using plasma ALP as an indicator of sex, 1.6% of males would be misidentified as female while 8.1% of females would be misidentified as males. A summary of the results of this exercise

for E2, ALP and VTG in the captive brown trout are presented in Figures 3-5. The extent of overlap between male and female E2 levels is clearly evident, particularly during the early part of the year (Fig. 3(a)). In Figure 3(b), the relative proportions of male and female fish likely to be misidentified at each sample time, if a cut-off is employed, are depicted. Comparison of the two graphs clearly shows that the likelihood of accurately identifying male or female fish increases as the separation between the E2 levels observed in the two sexes increases. For E2, there is a high expectation of misidentifying the sex of the fish from February through to July with an uncertainty approaching 50% for females during March. Highest confidence of an accurate identification is present during the period August to November where there is complete separation of the male and female distributions.

For ALP, considerable overlap of the range of levels in male and female fish is evident throughout the period February to June (Figure 3(a)) and this is reflected by high uncertainty ($\leq$ 50%) associated with the identification of female fish by ALP during this period (Figure 3(b)). Even during the period July to November where divergence between plasma ALP in the two sexes was greatest, confidence in identifying females based on ALP measurement did not reach 100%.

For the VTG data, there was considerable divergence between the levels in male and female plasma throughout the period of the study. There is no overlap between the range of measured values for male and female fish at any time point (Figure 5(a)), although the tails of each distribution, calculated on the basis of the sample measures, do crossover. Consequently, the data presented in Figure 5(b) indicate that VTG presents a low risk of misidentification, even during the period February – May when, for ALP and E2, the likelihood of misidentification was highest.

Comparison of markers of sex

Blood samples were analysed from a total of 241 salmon and sea trout that were trapped while moving upstream in the River Lune (Environment Agency Forge Trap, Halton, Lancashire, U.K.). Between April and July the catch consisted primarily of sea trout. Between July and October, salmon predominated (salmon: mean 3255 ± 104 g; range 1250 – 7190 g; sea trout: mean 2275 ± 78 g; range 1200 – 6100 g).

Plasma E2, ALP and VTG in upstream migrating salmon and sea trout

Plasma levels of E2, ALP and VTG were determined for each fish and sex was apportioned on the basis of plasma VTG levels (see below for details). There were some differences in the profiles of E2, ALP and VTG in the two species of fish. In female sea trout during the period April to August there was a downward trend in plasma E2 levels, from c. 2.0 ng ml^{-1} during spring to a mean of 0.7 ± 0.2 ng ml^{-1} in July, whereas in female salmon there was a very pronounced increase in plasma E2 between August (0.1 ± 0.1 ng ml^{-1}) and October (5.1 ± 0.8 ng ml^{-1}). In female sea trout mean plasma concentrations of both ALP and VTG remained relatively stable throughout the sampling period (20 – 50 μg ml-1; 25,000 – 35,000 μg ml^{-1} respectively) whereas in contrast, in female salmon both ALP and VTG levels increased coincident with the elevation in plasma E2. In males of both species levels of E2, ALP and VTG remained very low throughout the study period.

Discrimination of sex in upstream migrating salmon and sea trout using plasma E2, ALP and VTG levels

Plasma E2 levels failed to provide a reliably discriminatory signal for the sea trout and salmon, other than for the late sampled salmon, in which divergence between males and females was most marked (Fig. 6(b)). In distinct contrast to the data collected from the captive brown trout population, plasma ALP appeared to provide a more informative marker of sex in the trapped fish. Among the sea trout, no male (identified using plasma VTG) exhibited plasma ALP levels greater than 6.5 μg ml^{-1} and no female exhibited plasma ALP levels lower than 13.2 μg ml^{-1} (Fig. 7(a)) A similar level of discrimination was also evident for the salmon data, although the thresholds were shifted (Fig. 7(b)). Plasma ALP levels did not exceed 12.6 μg ml^{-1} in male salmon and were no lower than 15.4 μg ml^{-1} in female salmon. As was evident for the captive brown trout population, plasma VTG levels exhibited the greatest level of divergence and were the most reliable identifier of sex. The sea trout data segregated into two clear groups; one whose plasma VTG levels did not exceed 10 μg ml^{-1} and a second group whose plasma VTG levels were no lower than 800 μg ml^{-1} (Fig. 8(a)). Similarly for the salmon, there was clear divergence between fish that displayed plasma VTG levels no greater than 0.7 μg ml^{-1} and those that were characterised by plasma VTG levels no lower than 6975 μg ml^{-1} (Fig. 8(b)).

Discrimination of sex in upstream migrating salmon and sea trout using the "Best Checker" immunochromatographic vitellogenin detection kit

Complete (100%) agreement was obtained between samples in which VTG levels measured by ELISA were <100 μg ml^{-1} and samples which tested negative for VTG using the "Best

Checker" test sticks (Fig. 9). It should be noted that the batch of test kits used for this phase of the study were from the production run and were supplied with a proprietary dilution buffer which was used during the evaluation whereas the kits used for the captive brown trout study were pre-production. The manufacturers recommended dilution for applying plasma to the test stick is 1 in 500. In order to confirm that this was an appropriate dilution for use with *Salmo* species a number of samples from the Forge Trap fish covering a range of VTG concentrations was tested at 1 in 10, 1 in 100 and 1 in 500 dilutions. At a dilution of 1 in 10 males with plasma VTG levels of approximately 1 μg ml^{-1} as determined by ELISA gave positive responses. At dilutions of 1 in 100 this problem of misidentification of males was eliminated but at this dilution females at the higher end of the scale were found to register with possible false negatives. However, at a plasma dilution of 1 in 500 females with VTG concentrations as high as 93000 μg ml^{-1} provided an unequivocal positive result. The manufacturers recommended protocol was therefore adopted and with the production run kits this eliminated false positive and negative results. The frequency of disagreement between sex ascribed by visual inspection at the time of trapping and that inferred from VTG measurement or "Best Checker" testing is presented in Table 1.

DISCUSSION

INDICATORS OF SEX IN CAPTIVE BROWN TROUT

The experimental population of brown trout was exposed to a natural temperature and photoperiod regime and held in pristine Windermere lake water. The time-course of changes in somatic and endocrine parameters in the captive population of brown trout during sexual

maturation were consistent with those previously reported for this species (Kime and Manning 1982, Norberg *et al*. 1989). Inspection of plasma T levels and gonadosomatic indices confirmed that gonadal development progressed in synchrony in both males and females. We therefore concluded that the captive population of brown trout displayed a reproductive development profile comparable to that of a free-living population at this latitude.

Mean plasma E2 levels were for most of the year significantly higher in female fish than in males but there was a high level of inter-individual variation, resulting in considerable overlap between the ranges of plasma E2 levels for the two sexes. There was therefore a high degree of uncertainty when attempting to ascribe the sex of an individual based only on plasma E2 level, most markedly in terms of the potential for misidentifying females with E2 levels in the lower end of the range as males. The likelihood of misidentification was as high as 50% in the early stage of the cycle. This finding contrasts with previous work on wild populations of the white sturgeon (*Acipenser transmontanus* Richardson) in which plasma levels of the steroids T and E2 were both reliable predictors of sex in mature fish (> 96% correct identification; Webb *et al*., 2002). In a second study on white sturgeon, E2 was found to be wholly unsuitable as an indicator of sex in fish up to the age of 36 months (Feist *et al*., 2004) whereas androgens were effective indicators of sex within 18 months in some populations. Clearly, the species and context in which measurements are to be made has a strong influence on selection of the most appropriate indices.

The concentration of plasma ALP was also found to be an unsatisfactory predictor of sex in the captive brown trout. When trying to ascribe sex to individual fish, the use of plasma ALP levels presented as high a degree of uncertainty (<50%) as E2. Nonetheless, plasma ALP has

successfully been used to discriminate between sexes in galjoen (*Coracinus capensis* Cuvier; van der Lingen & Cook, 1990) and despite the inadequacy of plasma ALP as a marker of sex in the captive brown trout there was a high degree of correlation between individual plasma VTG levels and plasma ALP levels, supporting the assumption that the ALP assay was providing a surrogate measure of VTG concentration and confirming similar findings by others (Verslycke *et al*., 2002).

Plasma VTG was the most reliable marker of sex in the captive brown trout population with clear divergence between males and females for both for the mean levels at each sample time and the values for individual fish. These differences were clear even during the early phase of the reproductive cycle, meaning that the sex of individual fish could be identified with a high degree of certainty up to 9 months in advance of spawning. Perhaps surprisingly, in the light of previous findings in striped bass (*Morone saxitilis* Walbaum; Kishida *et al*., 1992), greater amberjack (*Seriola dumerili* Valenciennes; Takemura *et al*., 1996) and coho salmon (*Oncorhynchus kisutch* Walbaum; Gordon *et al*., 1984) we were unable to detect VTG in samples of superficial mucus, even in cases where blood levels of VTG were very high. In addition to the direct measurement of plasma VTG levels by ELISA, plasma samples were also screened for the presence or absence of VTG using the "Best Checker" VTG test kit. The preliminary results obtained were encouraging and these were therefore deployed in the second phase of the study.

INDICATORS OF SEX IN UPSTREAM MIGRATING SEA TROUT AND SALMON

The period during which upstream migrating salmonid fish were sampled at the R. Lune Forge trap comprised two phases; a run of sea trout between April and August, with peak

numbers of fish being trapped during June, and a run of Atlantic salmon between July and October, with most fish being trapped between September and October. The sea trout displayed few, if any, secondary sexual characters and this made identification of the sex of the fish by visual inspection at the time of capture difficult. In contrast, secondary sexual characters were more evident among the salmon, particularly during the later stages of the run.

Plasma E2, VTG and ALP in the migrating sea trout and salmon

Plasma E2 levels in the female sea trout declined during the period in which fish were trapped and plasma ALP and VTG levels in the female sea trout were considerably higher than those in the captive female brown trout over the same period. The data from the trapped sea trout were strongly suggestive of these fish having reached, in April, a stage of reproductive maturity not evident among the captive brown trout until August. Whether this relates to the very different life histories of the captive and wild populations, or to age, or some other factor is unclear. The E2, VTG and ALP profiles for the salmon were very different to those for the sea trout and suggested that vitellogenesis in the salmon was at an earlier stage than in the sea trout as the fish entered freshwater. Comparison of the data collected from salmon with those from the captive brown trout population suggests that the reproductive cycle in the salmon was aligned more closely to that of the captive brown trout.

Discrimination between males and females among the trapped sea trout and salmon

The external morphology of each individual fish was used to infer the sex of the individual at the time of capture by the EA fisheries personnel operating the trap. The sex of each fish

derived from this visual inspection was compared to that informed by plasma VTG measurement. Unsurprisingly, because of the greater frequency of readily identifiable secondary sexual characters, a much higher degree of accuracy was achieved in identifying the sex of upstream migrating salmon by visual inspection than was the case for sea trout. Given that most of the sea trout run took place in spring and early summer whereas the majority of the salmon were trapped during late summer and autumn the obvious conclusion is that reproductive development was further advanced in the later running fish than those running earlier in the year. This is not entirely consistent with the E2 and VTG data. These suggested that vitellogenesis was at its peak, or declining, in the trapped sea trout while in the salmon trapped during July and August the vitellogenic process was in its upward phase. However, we did not measure male-specific elements of the reproductive system, such as plasma levels of 11-ketotestosterone, which may have been more reflective of the frequency of secondary sexual characters that were noted. It is also possible that the extent to which secondary sexual characters are retained by males between reproductive periods differs between salmon and sea trout. It was also noted that there was greater divergence in the range of individual plasma ALP levels in the trapped sea trout and salmon between individuals identified as male and female than was the case for the captive brown trout population. This observation suggested that plasma ALP may be a more reliable indicator of sex in wild-caught salmonids than was suggested by the results of the captive brown trout study. The discrepancy may be related to the conditions under which the brown trout were held. Male and female fish were not segregated and it is possible that either sex received inappropriate exposure to water-borne androgens or estrogens excreted by the other.

Use of the "Best Checker" VTG test kit to screen trapped fish

The results obtained when plasma samples collected from the trapped fish were screened for VTG using the "Best Checker" kit were consistent. All those fish for which VTG levels were measured as 10 μg ml^{-1} or less tested negative with the kit while all those fish whose VTG levels were 800 μg ml^{-1} or greater tested positive. In practice this meant that all those fish identified as male on the basis of direct measurement of VTG levels in plasma were also identified as male using the "Best Checker" kit.

CONCLUSIONS

These data suggest that continued reliance on the visual identification of sex by using morphological differences will lead to inaccuracy in the assessment of the relative numbers of male and female sea trout migrating upstream during spring/summer. This problem of misidentification is less pronounced for late summer/autumn migrating salmon. It is recommended that one of the biochemical methods employed in this study be adopted, until such time as a genetic marker of sex is available for *S. trutta* and *S. salar*. Direct analysis of plasma VTG levels provides high confidence that sex can be identified accurately and provides precise data on the levels of VTG circulating in the blood of sampled fish at the time of capture. This might have diagnostic value with regard to detecting alterations in the timing of the reproductive cycle in addition to fulfilling the requirement for identifying the sex of the individual fish. The combination of moderate cost and added informational content make this an effective option. The "Best Checker" immunochromatographic test method is more limited in scope but may be operationally more easily implemented. The indirect assessment of VTG by measurement of ALP did not provide particularly effective sex discrimination during the preliminary study. However, when applied to samples collected from trapped fish this method performed quite well and may therefore justify further evaluation.

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Table 1. The total number of sea trout and salmon trapped on the R. Lune for which blood VTG analyses were carried out, the number of fish that were identified incorrectly as females or males, and the number whose sex was correctly identified at the time of trapping.

	Species	
	Sea trout	Salmon
Total number of fish	129	110
Males identified as females	11 (8.5%)	1 (0.9%)
Females identified as males	18 (14%)	3 (2.7%)
Correctly identified	100 (77.5%)	106 (96.4%)

Figure Legends

Figure 1. Mean plasma ALP (a) and VTG (b) levels in male (●) and female (○) brown trout sampled at two-weekly intervals. Each point represents the mean ± SEM, n = 4 – 16. Total number of fish at each sample point = 20. Significant differences between male and female fish are denoted by: *** $P<0.001$. NSD: no significant difference.

Figure 2. Normal distributions for plasma ALP levels in male and female brown trout sampled on 29.5.01 indicating the extent of overlap and areas of uncertainty.

Figure 3. (a) Plasma levels of E2 in individual brown trout sampled at two-weekly intervals between February 2001 and March 2002. The solid line represents the point at which the calculated distributions for male (●) and female (○) fish intersect. (b) The proportion of male and female fish likely to be misidentified if the crossover depicted in (a) is used to discriminate between male and female fish on the basis of plasma E2 levels.

Figure 4. (a) Plasma levels of ALP in individual brown trout sampled at two-weekly intervals between February 2001 and March 2002. The solid line represents the point at which the calculated distributions for male (●) and female (○) fish intersect. (b) The proportion of male and female fish likely to be misidentified if the crossover depicted in (a) is used to discriminate between male and female fish on the basis of plasma ALP levels.

Figure 5. (a) Plasma levels of VTG in individual brown trout sampled at two-weekly intervals between February and October 2001. The solid line represents the point at which the calculated distributions for male (●) and female (○) fish intersect. (b) The proportion of

male and female fish likely to be misidentified if the crossover depicted in (a) is used to discriminate between male and female fish on the basis of plasma VTG levels

Figure 6. Plasma E2 levels in individual (a) sea trout and (b) salmon caught at the Forge trap on the R. Lune during 2002. Fish are identified as male (●) or female (○) based on the morphological assessment made at the time of capture.

Figure 7. Plasma ALP levels in individual (a) sea trout and (b) salmon caught at the Forge trap on the R. Lune during 2002. Fish are identified as male (●) or female (○) based on the morphological assessment made at the time of capture.

Figure 8. Plasma VTG levels in individual (a) sea trout and (b) salmon caught at the Forge trap on the R. Lune during 2002. Fish are identified as male (●) or female (○) based on the morphological assessment made at the time of capture.

Figure 9. The results of testing plasma samples collected from sea trout and salmon at the Forge trap on the R. Lune with the "Best Checker" VTG test kit. All samples that tested positive for VTG are denoted by ○ while those testing negative for VTG are denoted by △. (a) The result for each fish obtained using the "Best Checker" kit plotted against the VTG level for each fish as determined by ELISA. (b) Plasma VTG levels for all salmon and sea trout, indicating whether the sample tested positive or negative for VTG using the "Best Checker" kit.

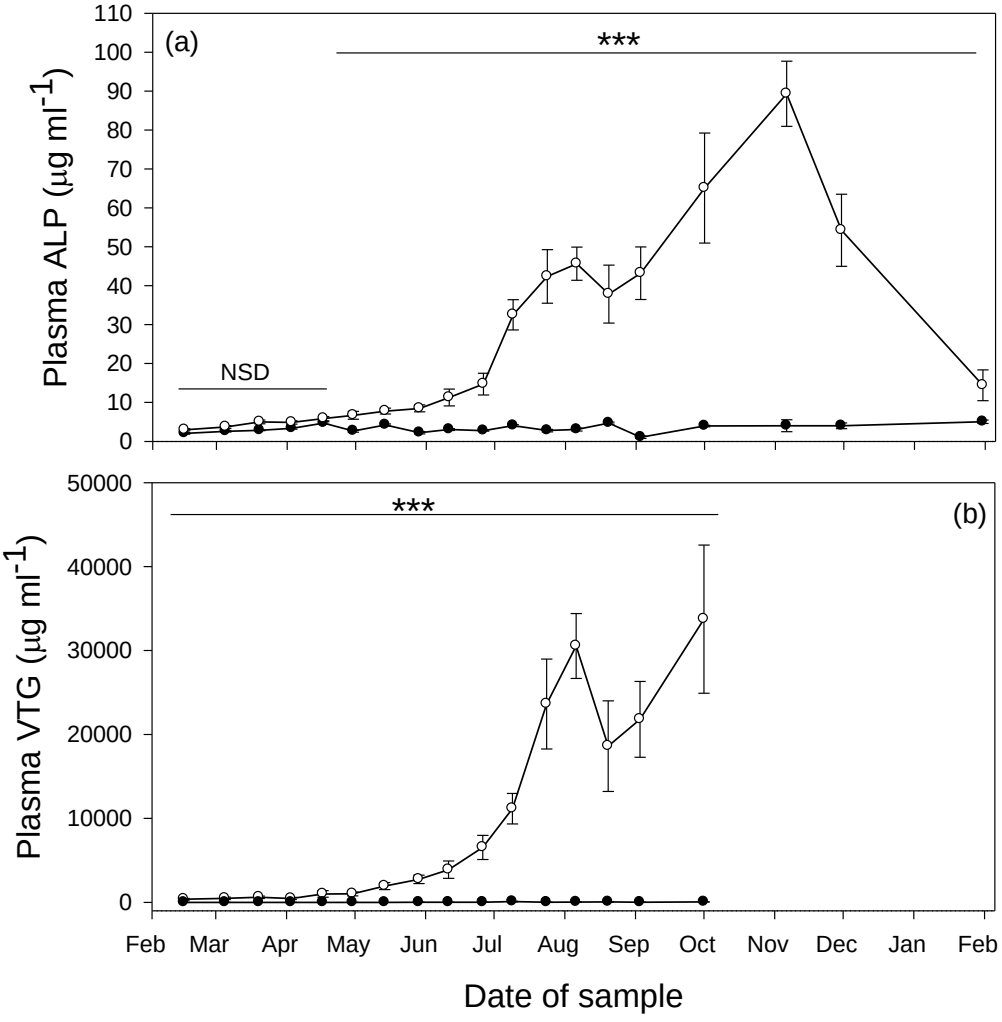


Figure 1. Pottinger, Pulman, Carrick & Scott

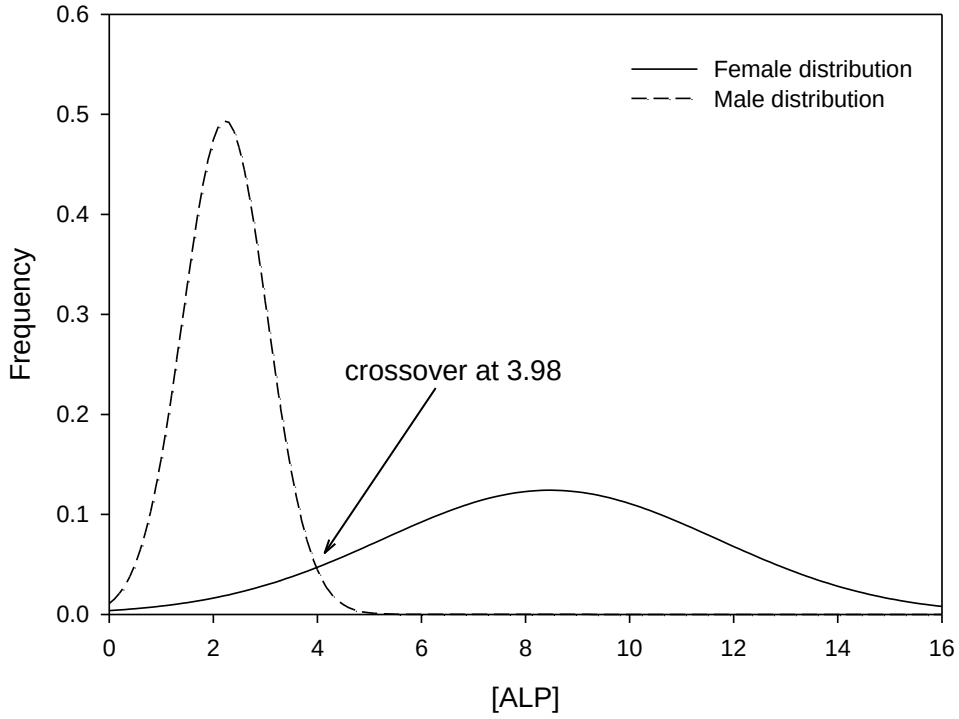


Figure 2. Pottinger, Pulman, Carrick & Scott

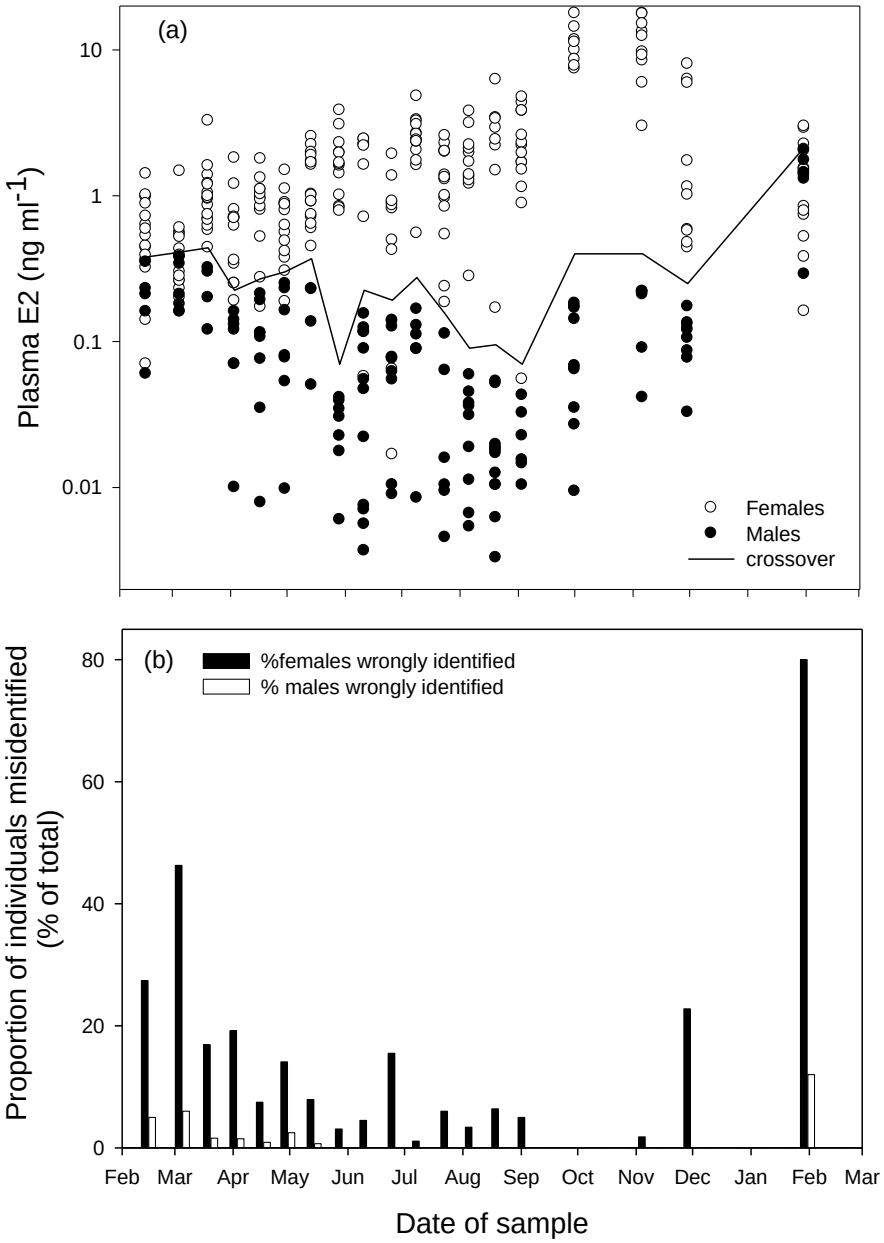


Figure 3. Pottinger, Pulman, Carrick & Scott

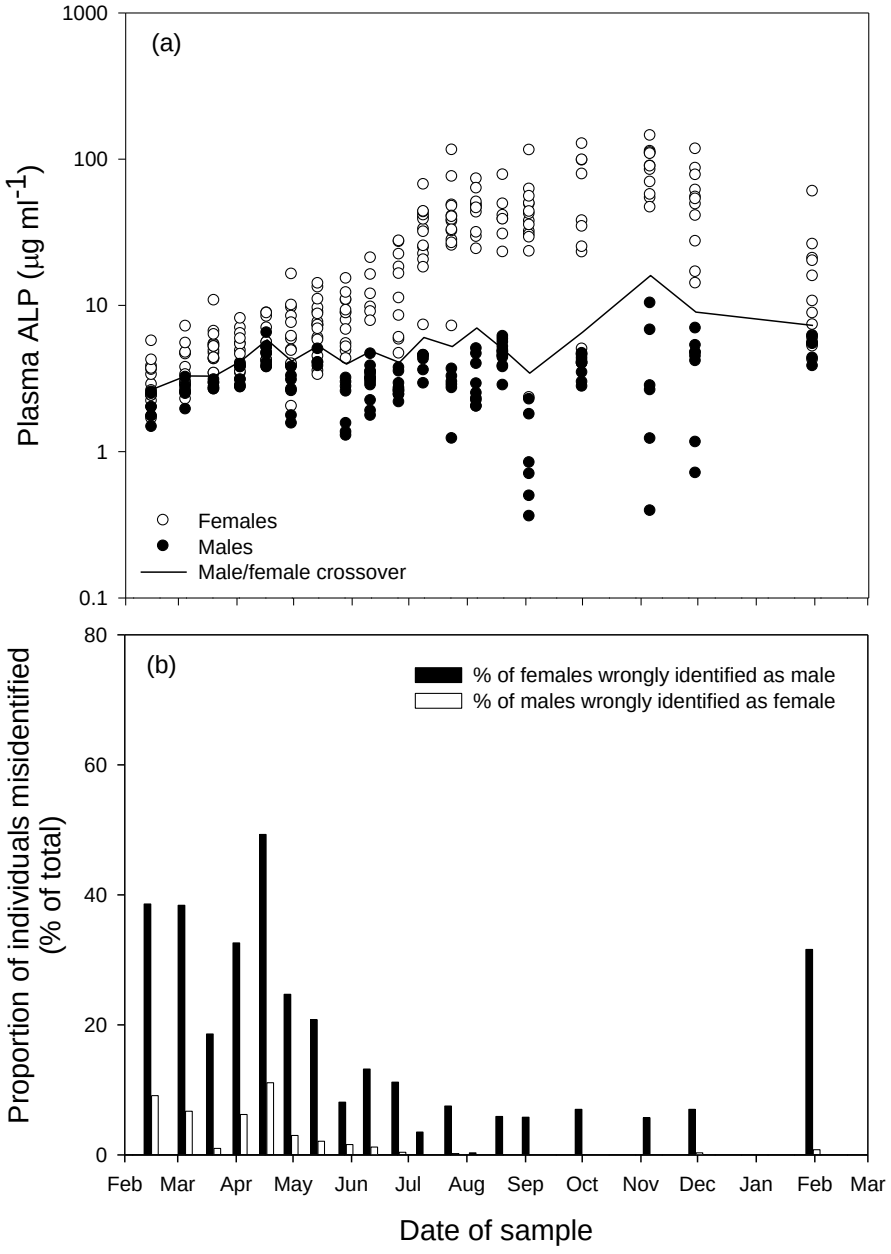
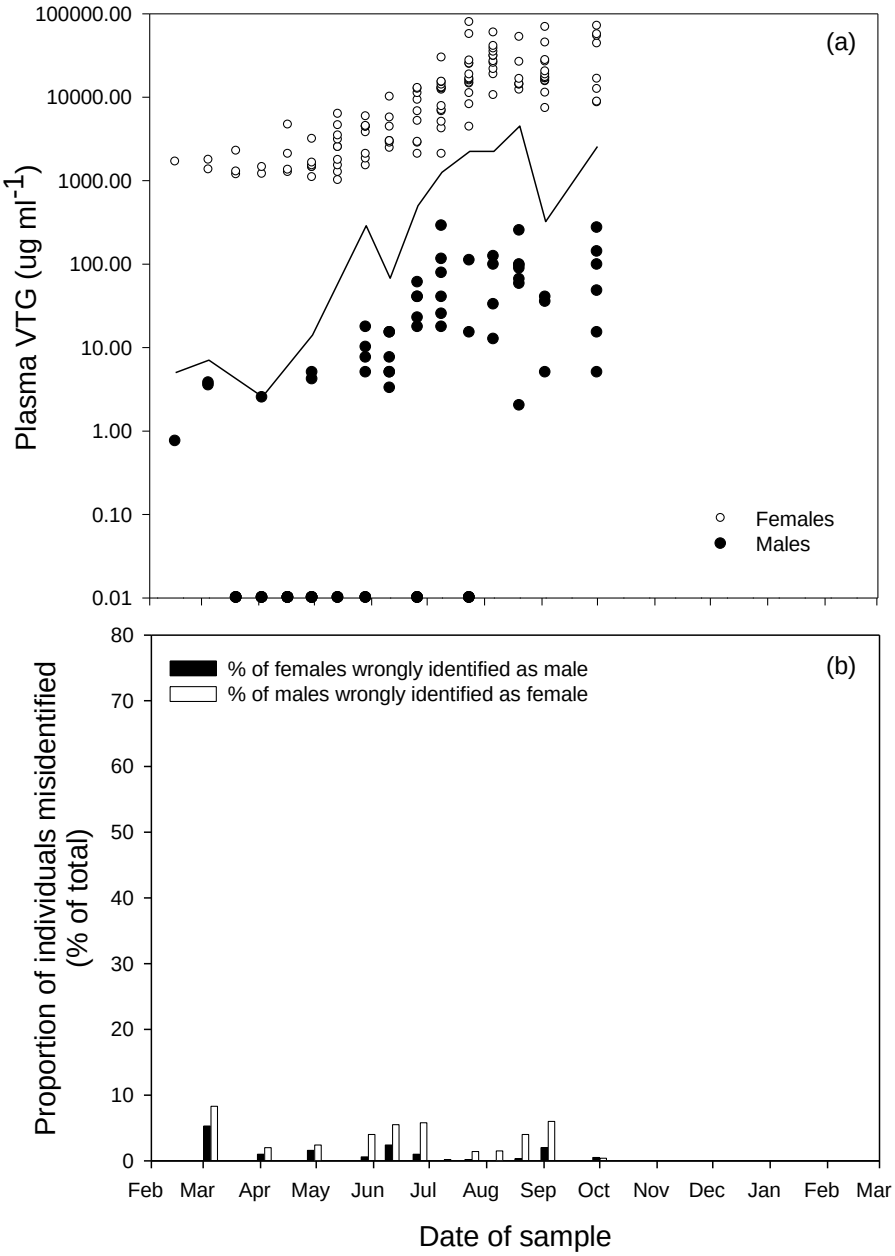


Figure 46. Pottinger, Pulman, Carrick & Scott



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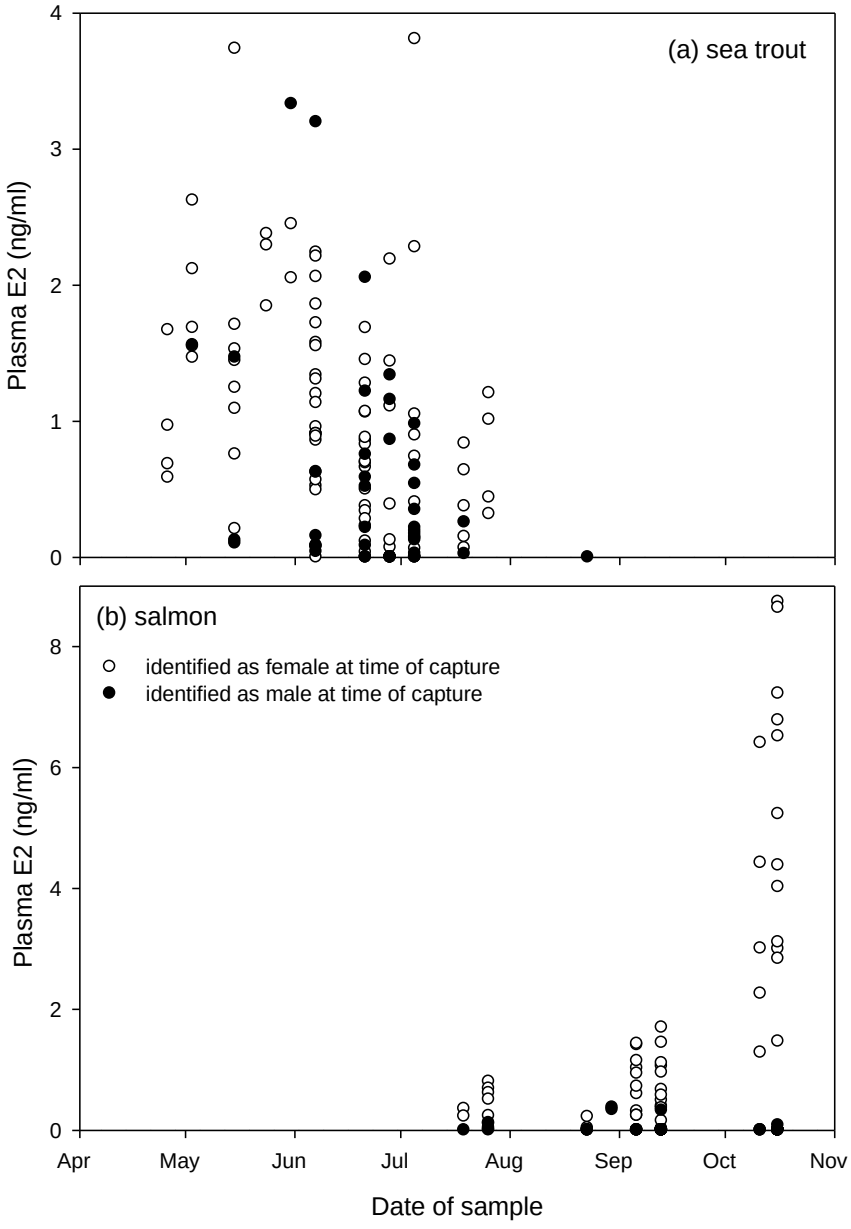

Figure 6. Pottinger, Pulman, Carrick & Scott

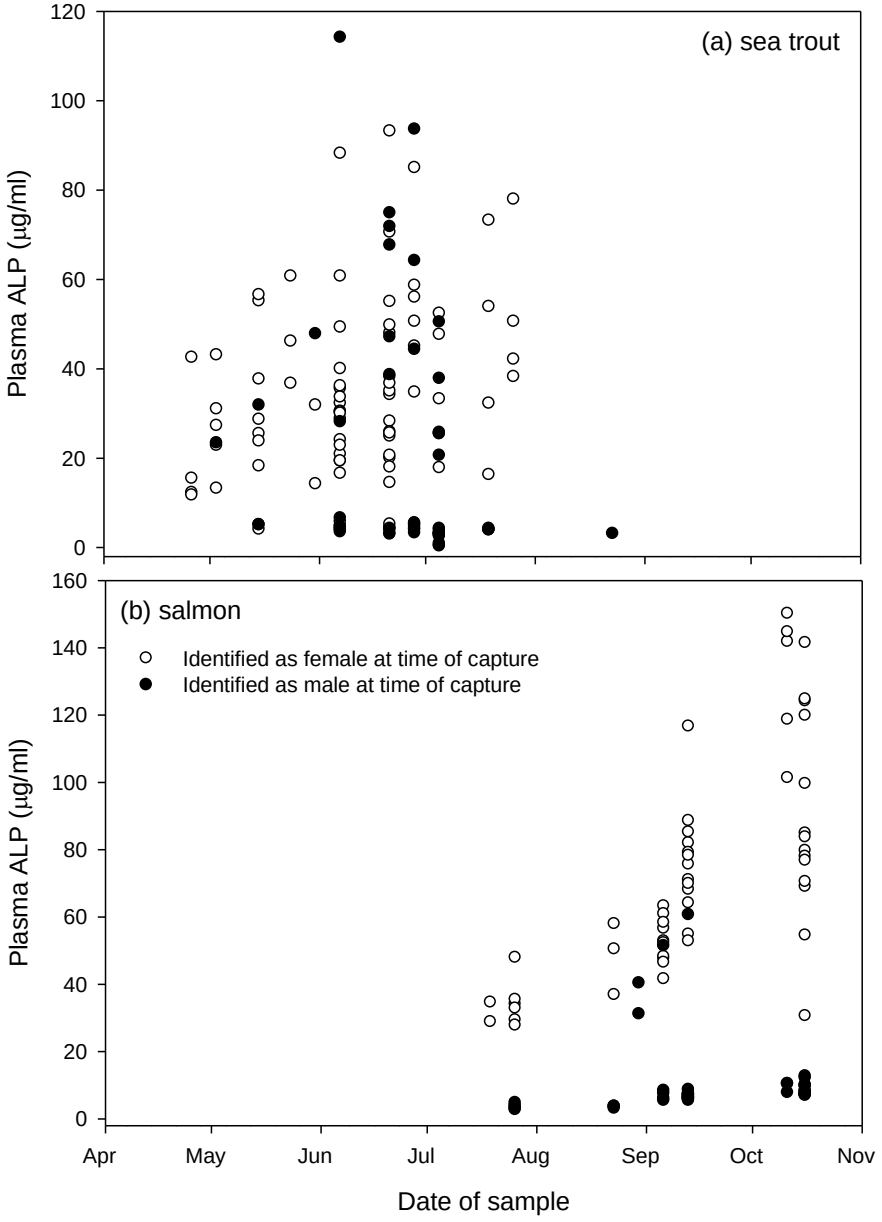

Figure 7. Pottinger, Pulman, Carrick & Scott

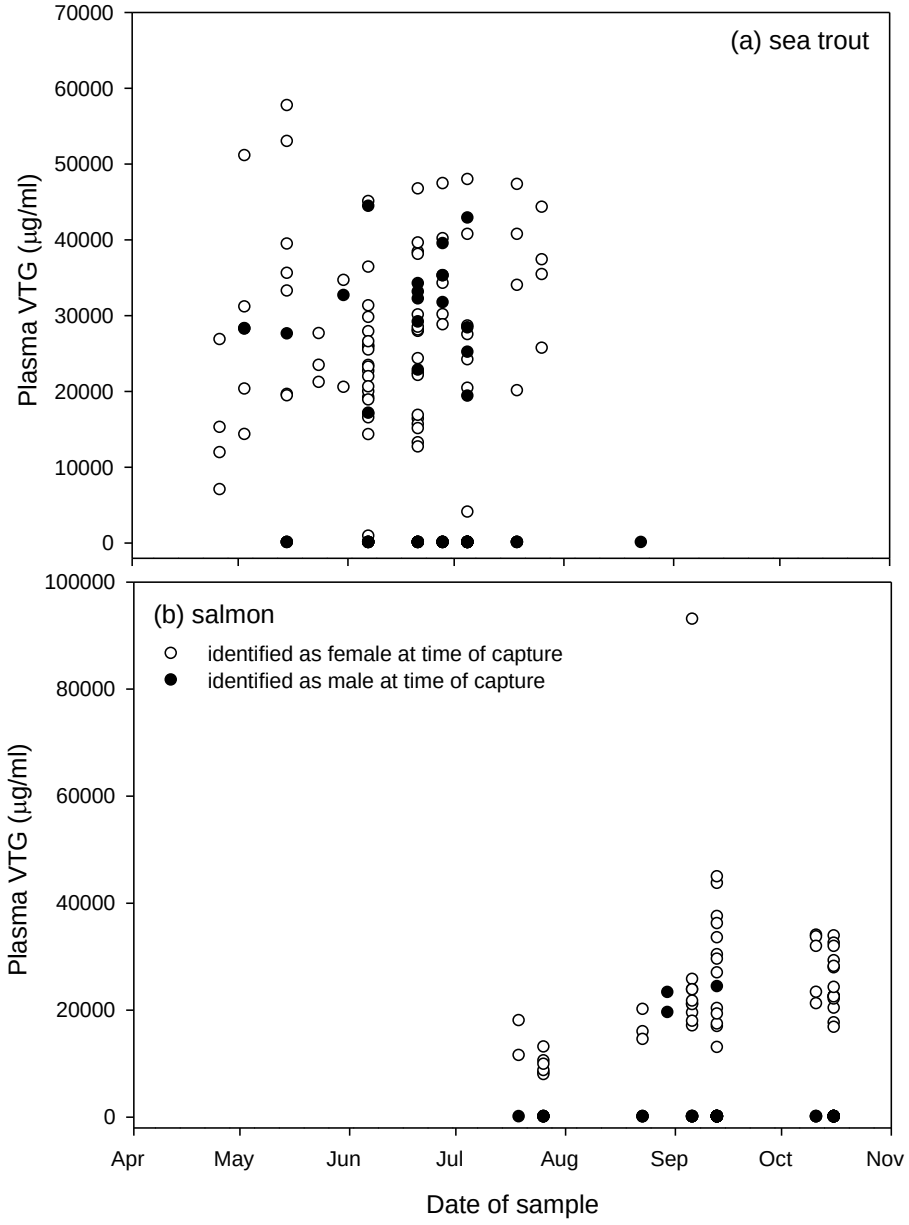

Figure 8. Pottinger, Pulman, Carrick & Scott

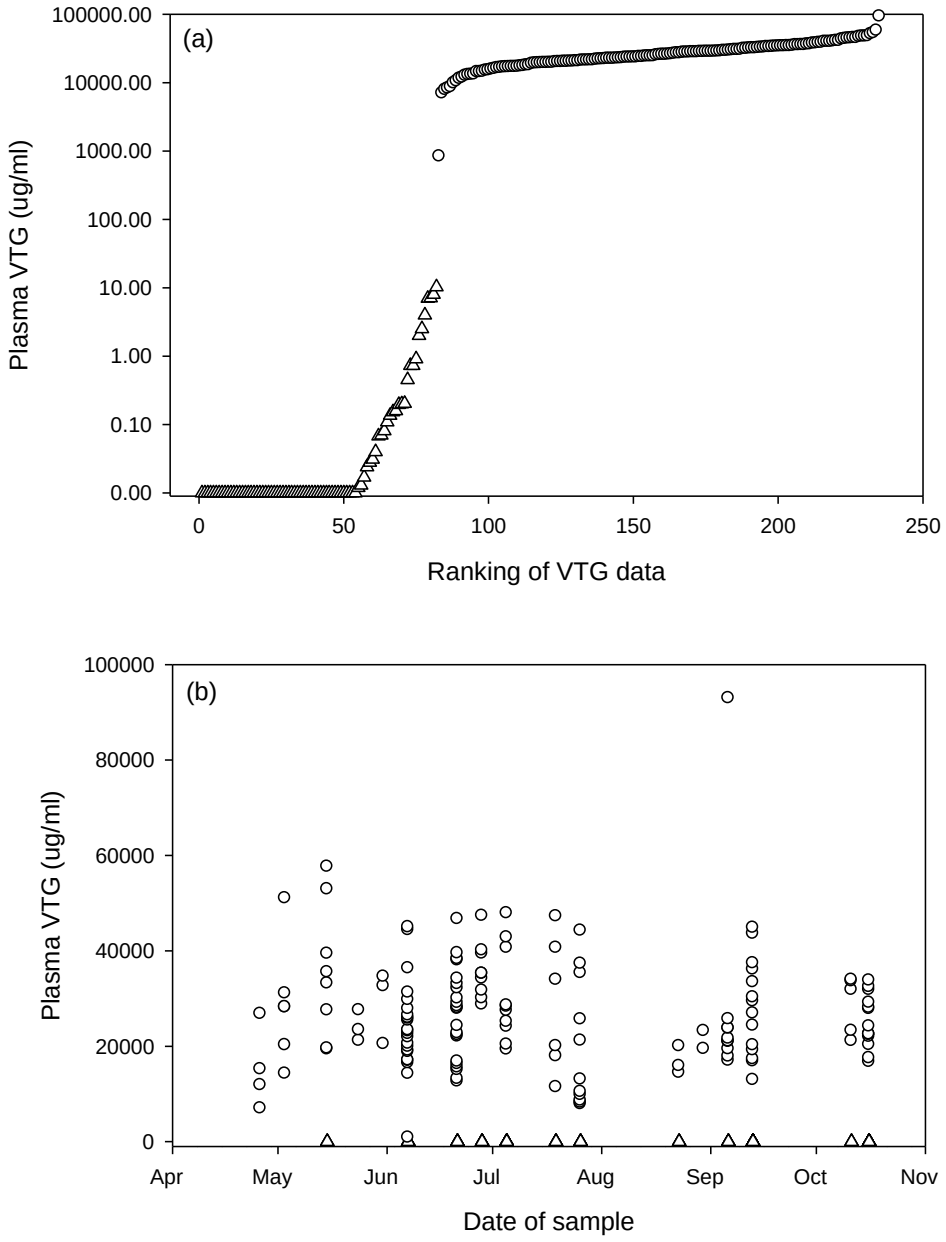

Figure 9. Pottinger, Pulman, Carrick & Scott