# Ejaculate expenditure and timing of gamete release in rainbow trout *Oncorhynchus mykiss*

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Using a novel methodology, natural ejaculate volumes of 14 male rainbow trout *Oncorhynchus mykiss* were compared when males were housed with one female (absence of a rival male) or with another male and one female (rival male present). Contrary to theoretical predictions, male ejaculate expenditure was not influenced by the presence of a rival male. Male gape duration was positively correlated with the volume of sperm ejaculated. Release of sperm by the male always preceded release of eggs by the female. Analysis of the timing and duration of ejaculation suggests that males may rely on the timing and proximity of gamete release to enhance fertilization success. These results are discussed in the context of sperm competition theory. © 2008 The Authors

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## INTRODUCTION

Sperm competition occurs when sperm from ejaculates of multiple males compete to fertilize eggs from a single female (Parker, 1970). Theory suggests that sperm compete numerically, with increasing sperm number leading to increasing fertilization success (Parker, 1982, 1990*a*, *b*), a notion that has received empirical support in externally fertilizing fish species (Marconato *et al.*, 1995; Marconato & Shapiro, 1996; Hoysak & Liley, 2001; Liley *et al.*, 2002). Because sperm production can be costly (Dewsbury, 1982; Olsson *et al.*, 1997) and sperm reserves can be depleted over successive mating attempts (Nakatsuru & Kramer, 1982; Preston *et al.*, 2001; Ambriz *et al.*, 2002), males are expected to strategically allocate available sperm reserves (Parker, 1982, 1990*a*, *b*; Parker *et al.*, 1996). Specifically, males are expected to ejaculate a minimal amount of sperm when mating in the absence of male–male competition, but increase ejaculate expenditure when spawning in the presence of a single rival male (Parker, 1998).

Recent empirical studies have demonstrated that males are indeed sensitive to the presence of rival males (Wedell *et al.*, 2002) and in a variety of species, from

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several taxa, males ejaculate more sperm when the perceived risk of sperm competition is high (*e.g.* mating with a female in the presence of a single rival male) than when the perceived risk of sperm competition is low (mating with a female with no male competitors present, *e.g.* insects: Gage & Barnard, 1996; Schaus & Sakaluk, 2001; fishes: Pilastro *et al.*, 2002; Evans *et al.*, 2003; Zbinden *et al.*, 2003; birds: Pizzari *et al.*, 2003; mammals: Pound & Gage, 2004), although this is not always the case (insects: Schaus & Sakaluk, 2001; amphibians: Byrne, 2004). Yet in fishes, only recently have attempts been made to measure the amount of sperm released at a spawning or mating event, as opposed to estimates of potential ejaculate expenditure based on relative testes mass (measured using the gonado-somatic index, $I_G$; Stockley *et al.*, 1997) or the amount of milt (sperm and seminal fluid) extracted by 'stripping' (abdominal massage) males manually (Gage *et al.*, 1995). Ejaculate expenditure has been measured in internally fertilizing species where sperm are released in bundles (spermatozeugmata) allowing collection from the females genital tract following sperm transfer (Evans & Magurran, 1999). Determination of ejaculate expenditure in externally fertilizing species, however, is more difficult due to the rapid dilution of milt following ejaculation. Techniques developed to measure the number of sperm released into the surrounding water have allowed researchers to estimate the numbers of sperm ejaculated in coral reefs (Shapiro *et al.*, 1994; Marconato *et al.*, 1995; Marconato & Shapiro, 1996) and fishes spawning in aquaria (Fuller, 1998; Pilastro *et al.*, 2002; Zbinden *et al.*, 2003, 2004), but studies detailing the amount of sperm ejaculated in fast flowing water have been lacking.

In Pacific salmonids, genus *Oncorhynchus*, following migration to a suitable spawning area, females select and defend a territory, building a series of nests. A dominant male attempts to monopolize access to the female, directing aggressive actions towards subordinate and precocious (sneaker) males. Spawning areas are typically teeming with conspecific males and both subordinate and precocious males may attempt sneak fertilizations, potentially leading to intense levels of sperm competition (Gross, 1985). A primary objective in the present study was to estimate the amount of sperm released during a spawning event by male rainbow trout *Oncorhynchus mykiss* (Walbaum). To meet this objective, a novel methodology was developed, using experimentally attached condoms, to collect an ejaculate in fast flowing water under semi-natural spawning conditions. A second objective was to examine the influence of behavioural competition and potential sperm competition on the amount of sperm released by a male. In addition, with the use of video records, the duration and relative timing of ejaculation in relation to oviposition were analysed in detail. This analysis was anticipated to reveal possible causal relationships between ejaculation and oviposition that may, in turn, influence the amounts of milt ejaculated at a single spawning event.

## METHODS

*Oncorhynchus mykiss* were obtained from a population of fish that move into the inlet to Pennask Lake, 50 km east of Merritt, B.C., Canada (Liley *et al.*, 1986). Spawning typically begins in early June and continues into early July. Fish were collected on 5 June 2003 and were transported to a laboratory at the University of British Columbia,

Vancouver, where males and females were placed in separate tanks supplied with a constant flow of dechlorinated city freshwater. The following day, each fish's fork length ($L_F$, mm) and body mass (g) were measured, and a small numbered tag was attached by threading a monofilament loop through the dorsal musculature behind the dorsal fin. Prior to all handling and sampling procedures, fish were anaesthetized in 0·05% 2-phenoxyethanol. At collection, all males were spermiating (sperm released under gentle pressure on the abdomen), whereas only unovulated (eggs not expressed during abdominal massage) females were selected.

## SPAWNING BEHAVIOUR

Sexual behaviour in *O. mykiss*, and other salmonids, consists of nest building by the female and courting by the male. Several detailed descriptions of the spawning behaviour of salmonids are available (Jones & Ball, 1954; Tautz & Groot, 1975; Satou *et al.*, 1991), so only a general description of the behaviour involved in spawning events is given.

Ovulated females select a nest site and dig a nest up to 150 mm deep. Females hover over the nest, periodically probing the gravel on the bottom of the nest with their anal fin, and directly before spawning crouch deep into the nest. A 'prespawning act', where the female remains in the nest and opens her mouth slightly (Jones & Ball, 1954; Satou *et al.*, 1991), usually follows crouching, and directly precedes the spawning act, except the female does not release eggs. In a 'spawning act', the female drops into the nest, accompanied by the male, and performs a 'gape' behaviour, a wide opening of the mouth (to an angle of *c.* 130°, Jones & Ball, 1954) while releasing eggs into the nest. Females then cover the nest with gravel.

Males, attracted to a nest-building female, perform 'attending' behaviour, in which a male maintains a position just behind, or to the side and behind, the female. From this position, the male periodically darts towards the female and performs a high frequency, low amplitude, body undulation, termed 'quivering'. The 'spawning act' in males involves the male rapidly entering the nest with the female and performing a 'gape' behaviour, similar to the above description in females, leading to sperm release.

## DETERMINATION OF SPERM CONCENTRATION

The concentration of sperm in milt (sperm and seminal plasma) is variable between males. Spermatocrit values (the proportion of packed sperm cells in a volume of milt) were used to provide a rapid approximation of sperm density in milt (Hoysak & Liley, 2001; Liley *et al.*, 2002) and to estimate the amount of sperm released during a spawning event, as there is a strong correlation between the number of sperm cells per unit volume and spermatocrit (Bouck & Jacobson, 1976; Tvedt *et al.*, 2001). Spermatocrit was measured by filling two microhaematocrit capillary tubes with milt and centrifuging in a microhaematocrit centrifuge for 10 min. Sperm cells pack down in the capillary tube forming an opaque layer below a clear layer of plasma. The spermatocrit for an individual was the average of the amounts of packed sperm in each sample, as measured using a haematocrit reader.

## COLLECTION AND MEASUREMENT OF EJACULATE AT SPAWNING

Milt ejaculated during spawning was collected in a latex condom attached to the male. Under anaesthetic, the anterior five fin rays of the anal fin were removed. A 50–70 mm piece of 10 mm diameter amber natural rubber latex tubing (Thermo Fisher Scientific, Waltham, MA, U.S.A.) with a groove cut along half the length of the tube, was stitched over the gonopore, with the uncut section of the tubing projecting below the base of the anal fin. Four threads held the tubing in place; one stitch was placed at

the anterior most portion of the tubing, 5 mm anterior to the genital papilla, two stitches were placed on the lateral portion of the tubing (one on the right-hand side and one on the left-hand side), *c.* 5 mm posterior from the genital papilla, and a final thread, placed through the basal articulation of the anal fin rays, was used to secure the tubing (Fig. 1, this procedure was approved by the UBC Animal Care Committee, Approval Number A99-0082). The flexible latex tubing fitted snugly against the fish and the seal was reinforced with a bead of Vetbond tissue adhesive (3M Canada Inc., London, Ontario, Canada) to prevent milt leakage. A condom (Trojan® latex condom; Carter-Horner Corp., Mississauga, Ontario, Canada) was tied tightly to the latex tube by a thread. The condom was attached immediately before the male was paired with a nesting female.

Surgery was performed at least 1 day before spawning trials and males were stripped of milt reserves at this time to avoid confounding the results by introducing variation in the amount of milt a male had stored at the beginning of the experiment. Olsen & Liley (1993) demonstrated that males rapidly replace milt stores when placed with a nesting female (within 60 min of being stripped of milt). In addition, data presented below indicate that males were not sperm limited at the time of the experimental spawning.

In all cases, males fitted with a condom readily spawned with a nesting female. Indeed, the attachment appeared to have no inhibitory effect on the male or female. Condoms were filled with *c.* 75 ml water prior to attachment to ensure that the condom remained open during spawning to allowing collection of the ejaculate. At spawning, the ejaculate flowed freely into the condom, with no visible milt leakage, and was diluted in the water originally placed in the condom. Spawning fish were under constant video surveillance [Sanyo SRT-500 VCR (Sanyo Electric Co Ltd, Tokyo, Japan) linked by MV45 Multivision Processor, Robot Research Inc. (Lumberton, NJ, U.S.A.) to four Sanyo B/W CCD cameras, VCB 3324]. Males were removed immediately after spawning and the condom was slipped off the latex tubing, while pinching the neck of the condom to prevent leakage. The contents of the condom were emptied into a 300 ml glass beaker and the total volume made up to 150 ml with water.
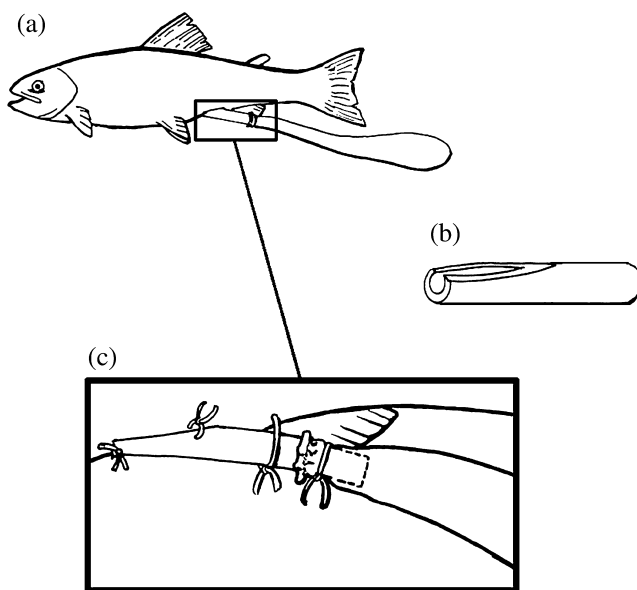


FIG. 1. (a) To collect sperm from male *Oncorhynchus mykiss*, a piece of rubber tubing was stitched to the ventral side of the male. (b) A U-shaped groove was cut out of the rubber tubing and it was securely placed over the gonopore. (c) A condom was attached to the posterior end of the tubing to collect ejaculated milt.

An estimate of the amount of packed sperm in the diluted milt was determined spectrophotometrically by measuring absorbance (Spectronic 21D, Milton Roy Co., Ivyland, PA, U.S.A.) at a wavelength of 410 nm (Billard *et al.*, 1971). A calibration curve was established using milt stripped from six males. Absorption was measured at six packed sperm concentrations in 150 ml water (Fig. 2). Two absorbance values were obtained at each dilution, and the mean absorbance values were used in the calibration curve. Absorbance values were plotted against sperm concentrations to create a standard curve (Fig. 2). It was not possible to reliably collect milt for spermatocrit determination once the latex tube was attached because sperm were diluted in the water originally placed in the condom. Therefore, estimates of the size of the ejaculate are given as amounts of packed sperm rather than a calculated volume of milt. Prior to all absorbance readings, the sperm and water mixture was mixed thoroughly to ensure a homogeneous sample and the reading was quickly recorded.

## EXPERIMENT 1: EJACULATE EXPENDITURE IN THE PRESENCE OF A RIVAL MALE

Each male was fitted with a condom and paired with a nesting female under two experimental conditions: alone and paired with a rival male ($n = 14$). Seven males were tested alone in the first trial and paired with another male in the second trial. The other seven males were tested in the reverse order. To ensure that the test male was always dominant and therefore most likely to spawn in the paired trials, pairs were selected in which the test male was largest of the two (dominant males: $n = 14$, mean $\pm$ S.E. mass $= 317\cdot0 \pm 11\cdot5$ g; subordinate males: $n = 8$, $268\cdot4 \pm 10\cdot4$ g). In the paired treatment, both males were fitted with a condom. The mean $\pm$ S.E. time interval between the end of a spawning and gamete collection was $106\cdot3 \pm 19\cdot7$ s.

Solitary males and dominant males in paired trials, directed courtship towards nesting females. The larger of the paired males quickly established dominance by directing aggressive behaviour (chasing and biting) towards the smaller male and monopolizing
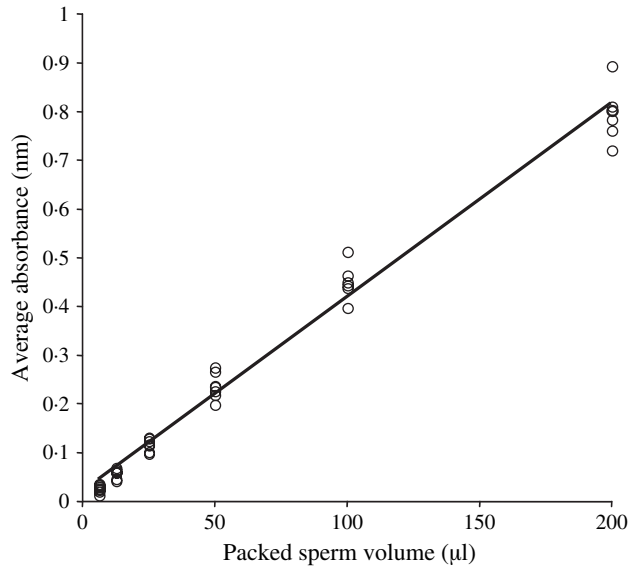


FIG. 2. Relationship for *Oncorhynchus mykiss* between packed sperm volume in the diluted milt and average absorbance as measured spectrophotometrically. The curve was fitted by: $y = 0\cdot017 + 0\cdot004x$ ($r = 0\cdot99$, $n = 36$, $P < 0\cdot001$).

access to the female. In the paired treatments, subordinates were largely excluded from the vicinity of the female. All solitary males and dominant males, but only two subordinate males, spawned.

Females ($n = 16$, $290 \cdot 2 \pm 6 \cdot 3$ g) were checked for ovulation every 2–3 days, and ovulated females placed in a separate holding tank. Twenty-four hours before a trial, females were placed in individually sectioned areas in an oval artificial spawning channel ($3000 \times 760 \times 600$ mm; details in Liley & Kroon, 1993). A gravel patch (*c.* $1200 \times 550$ mm, depth 50 mm) was constructed in the spawning channel. Submersible pumps maintained a constant flow of water. All spawning activity was confined to the gravel patch, facilitating constant video surveillance of a nesting pair.

The interval between the introduction of the male in the spawning channel and spawning, and the time from spawning to collection of ejaculate expenditure were recorded. The time from the start of the male gape to the start of the female prespawning act, the end of the female gape and the end of the male gape, and the duration of the male and female gapes were measured by frame-by-frame analysis of the video-tape (60 frames s$^{-1}$). It was not always possible to identify the onset and end of gaping in spawning fish, therefore the sample sizes for the behavioural observations listed in Table I are variable.

## EXPERIMENT 2: TIMING AND CHARACTERISTICS OF GAMETE RELEASE

Spawning was video-recorded from below to allow a detailed analysis of the physical position of spawning fish and the precise timing of gamete release. Two males were placed with an ovulated female in a spawning channel (1950 mm long, 450 mm wide and 450 mm deep) supplied with dechlorinated freshwater, with a constant flow maintained by submersible pumps (details in Liley *et al.*, 1986). A portion of the floor of the spawning channel was replaced by a transparent panel ($450 \times 450$ mm) and a gravel patch (*c.* $250 \times 450$ mm) was placed over this section to a depth of 50 mm. In several cases, a nesting female cleared an area over the panel, leaving a clear window through which spawning could be observed and recorded by video camera. Spawning was visible in four of 10 pairs established in the viewing chamber. In the other six pairs, spawning was obscured, either by gravel displaced by the female during digging or because the depth of the nest did not reach the transparent panel of the spawning channel.

The duration of sperm and egg release, and the interval between the onset of release of sperm and eggs were measured by frame-by-frame analysis (60 frames s$^{-1}$). The distance between male and female gonopores at the time of gamete release was measured by projecting the recorded image onto a large screen ($\times 4 \cdot 7$ original size) and marking the position of gamete release.

TABLE I. The timing and duration of gamete release by male and female *Oncorhynchus mykiss* in experiment 1. Mean $\pm$ S.E. and sample sizes (*n*) are shown for each variable

|  | *n* | Time |
|---|---|---|
| Interval between introduction of solitary male and spawning | 14 | $117 \pm 17$ min |
| Interval between introduction of dominant male and spawning | 14 | $124 \pm 19$ min |
| Male gape duration | 16 | $3 \cdot 42 \pm 1 \cdot 53$ s |
| Interval between start of male gape and female prespawning act | 7 | $0 \cdot 55 \pm 0 \cdot 31$ s |
| Duration of female prespawning act | 8 | $1 \cdot 27 \pm 0 \cdot 39$ s |
| Female gape duration | 8 | $1 \cdot 73 \pm 0 \cdot 19$ s |
| Interval between end of male gape and end of female gape | 7 | $0 \cdot 30 \pm 0 \cdot 21$ s |

## STATISTICAL ANALYSIS

Statistical analyses were performed with JMP (version 5.1, SAS Institute Inc., 2004). All data were normally distributed and paired *t*-tests were used to compare differences between treatments as the same male was used sequentially in each treatment. Linear regressions were used to examine correlations between variables. All other measurement are presented as means ± s.e. The level of significance was set at $P < 0.05$.

## RESULTS

### EXPERIMENT 1: EJACULATE EXPENDITURE IN THE PRESENCE OF A RIVAL MALE

There was no difference between the volume of sperm ejaculated in a male's first and second spawning (regardless of treatment, paired *t*-test, $n = 14$, $P > 0.05$) and there was no relationship between the time since a male was stripped of milt reserves and the volume of sperm ejaculated during spawning (first spawning, $r = 0.10$, $n = 14$, $P > 0.05$; second spawning, $r = 0.27$, $n = 14$, $P > 0.05$). Therefore, males did not appear to be sperm limited in this study.

There was considerable variation between males in the volume of sperm ejaculated in the two treatments, but the mean volume of packed sperm ejaculated by males when spawning in the solitary treatment ($92.1 \pm 30.3$ µl) was not significantly different from amounts released by the same males when in the presence of a competing male ($87.5 \pm 27.0$ µl; paired *t*-test, $n = 14$, $P > 0.05$; Fig. 3). There was also no difference between gape duration when males
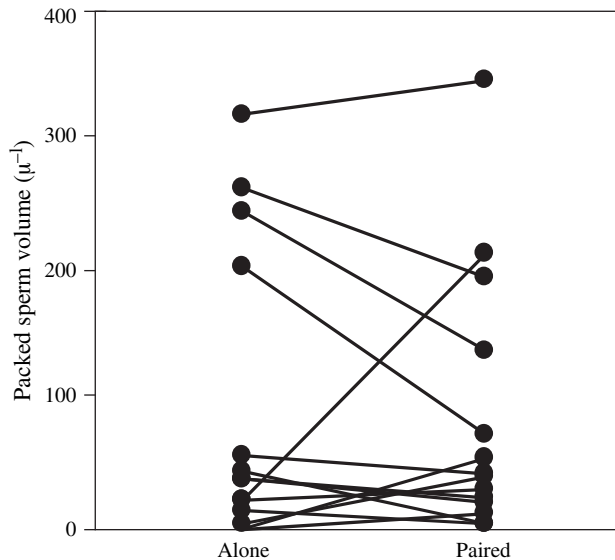


FIG. 3. Amount of packed sperm ejaculated when male *Oncorhynchus mykiss* spawned alone with a female or spawned when paired with another male. Male's ejaculate expenditure in each condition is connected by a line.

spawned alone or in the presence of a competing male (paired *t*-test, $n = 6$, $P > 0.05$), therefore, in subsequent analyses, all males were combined.

Male gape duration was positively correlated with the volume of packed sperm ejaculated (linear regression, $r = 0.59$, $n = 16$, $P < 0.05$; Fig. 4). The mean duration of the male's gape was just under 3.5 s (range 2–5 s; Table I) and male gape commenced *c.* 0.5 s before the female initiated the prespawning act, a behaviour that began directly after the male rapidly entered the nest. The female prespawning act lasted for just over 1 s, followed by a longer gape, *c.* 1.5–2.0 s. With the exception of one male whose gape continued 1.53 s after the female gape, male gape ended $0.01 \pm 0.15$ s after the female stopped gaping. The mean time between introduction to a nesting female and spawning did not differ between solitary and dominant males (paired *t*-test, $n = 14$, $P > 0.05$; solitary males: $117 \pm 17$ min; dominant males: $124 \pm 19$ min).

## EXPERIMENT 2: TIMING AND CHARACTERISTICS OF GAMETE RELEASE

While sperm ejaculation preceded oviposition in all cases, the interval between the onset of sperm and egg release was highly variable (Table II). For example, one male released sperm continuously for over 6 s before and during oviposition (Fig. 4). In two cases, subordinate males entered the nest after the dominant male had commenced ejaculation. One subordinate male began ejaculation before oviposition, the other male released sperm after the onset of oviposition. The average duration of oviposition was <1 s, with individual females varying little in the duration of egg release (Table II). The interval between the end of oviposition and sperm release averaged <0.25 s. At the
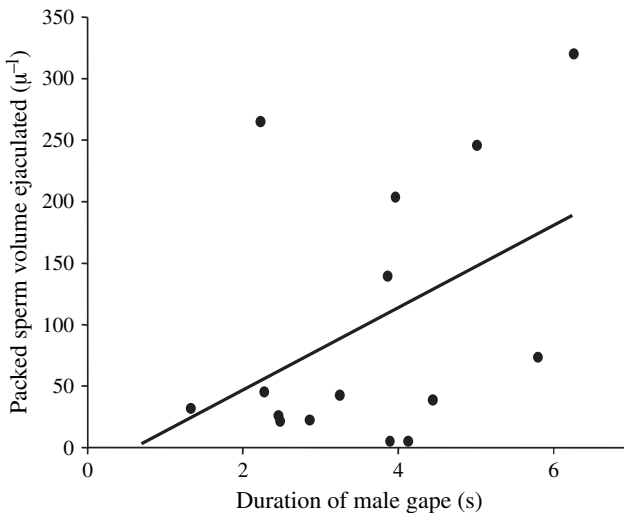


FIG. 4. Relationship between the duration of male *Oncorhynchus mykiss* gape and the amount of packed sperm ejaculated. The curve was fitted by: $y = 0.5532x - 19.964$.

TABLE II. The timing and duration of gamete release by male and female *Oncorhynchus mykiss* in experiment 2. Mean $\pm$ S.E. time and sample sizes ($n$) are given

|  | $n$ | Time (range) (s) |
| --- | --- | --- |
| Duration of sperm release | 4 | 2·43 $\pm$ 2·53 (0·80–6·16) |
| Duration of egg release | 4 | 0·83 $\pm$ 0·18 (0·67–1·10) |
| Interval between start of sperm and egg release | 4 | 1·67 $\pm$ 2·31 (0·30–5·13) |
| Interval between end of sperm and egg release | 3 | 0·24 $\pm$ 0·09 (0·18–0·35) |

time of gamete release, the gonopores of the spawning pair were 1·83 $\pm$ 1·45 cm apart ($n = 4$).

## DISCUSSION

A new methodology is described for measuring ejaculate expenditure in an externally fertilizing fish inhabiting fast flowing water. Contrary to theoretical prediction, there was no evidence that males facultatively increase ejaculate expenditure when spawning in the presence of a rival male. This result contrasts that of other externally fertilizing fishes where males adjusted their ejaculate size when facing an elevated risk of sperm competition (Fuller, 1998; Pilastro *et al.*, 2002; Zbinden *et al.*, 2003, 2004) but is similar to the results from the externally fertilizing frog *Crinia georgiana* (Tschudi), where ejaculate expenditure did not change as the number of competing males involved in the spawning increased (Byrne, 2004). Instead, the results of this study suggest that male *O. mykiss* may not rely on increased numbers of sperm to secure higher fertilization success when in competition with other males.

There may be several reasons why the results of this study do not support theoretical predictions. (1) The spawning arenas used in this study may not have provided accurate cues of sperm competition. (2) The sample size used in this study was too small to detect subtle differences in ejaculate allocation. (3) By using absorbance values instead of relying on sperm density counts, the results in this study may have been unknowingly influenced by urine or faecal matter. (4) Males may rely on enhancing sperm quality rather than manipulating sperm number in competitive trials. In competitive spawnings in Atlantic salmon *Salmo salar* L., sperm swimming speed predicts fertilization success (Gage *et al.*, 2004), while in the Australian field cricket *Teleogryllus oceanicus* (Le Guillou), males respond to increased sperm competition risk by increasing sperm viability (Thomas & Simmons, 2007). Thus, male *O. mykiss* may ejaculate sperm of higher quality that swims faster, rather than increasing sperm number, when spawning in the presence of a rival male. (5) Males may rely on the timing and location of sperm release to increase their fertilization success in competitive spawning situations, as males initiated their gape prior to the female's gape during spawning (experiment 1), and ejaculate sperm prior to egg release by the female (experiment 2). Therefore, dominant, courting males, which release sperm before rivals may enhance fertilization success, particularly as small differences in the timing of ejaculates can dramatically influence paternity (Yeates *et al.*, 2007). Furthermore, at the moment of gamete

release, the male and female gonopores were typically <30 mm apart. By releasing sperm in close proximity to the female gonopore prior to egg release, males can ensure rapid gametic association, which may enhance fertilization efficiency as gamete viability is short in salmonids (*c.* 30 s for sperm and *c.* 40 s for eggs; Ginsberg, 1963; Cosson *et al.*, 1995; Liley *et al.*, 2002) and, perhaps more importantly, fertilization occurs rapidly following sperm and egg interaction (Hoysak & Liley, 2001; Liley *et al.*, 2002).

During spawning, the first detectable sign of imminent gamete release was the onset of gaping by the male during an intense bout of probing and crouching by the female. Within 0·5 s, but in some cases longer, the female began the prespawning act, leading to gaping accompanied by oviposition. In all cases, the female prespawning act was preceded by male entry into the nest and male gape and sperm release ended only after the cessation of oviposition. This sequence of events strongly suggests that female gape and oviposition occur in response to male gape, as previously suggested in other salmonids (Satou *et al.*, 1991), although the opposite was suggested by Jones & Ball (1954). Once a male began ejaculation, he continued to release sperm until oviposition is complete. Dependence of the duration of male ejaculation upon the female's activity may account for the considerable variation in the amounts of ejaculate collected in experiment 1, in which a positive correlation between duration of male gape and amount of ejaculate collected was detected. Furthermore, males may gape and release milt alongside a nesting female without an accompanying gape and oviposition by the female, referred to as a 'false orgasm' (Jones & Ball, 1954; Satou *et al.*, 1991; Petersson & Järvi, 2001). By releasing sperm before eggs, males ensure that eggs are released directly into a cloud of milt and may enhance their success in competitive situations, but run the risk of releasing sperm when the female does not release eggs, potentially squandering gametic resources. The timing of gamete release suggests that females exert some control over reproduction simply by refraining from releasing eggs.

The novel methodology developed in this study for collecting ejaculates from an externally fertilizing fish spawning in fast moving water may have broad applications in future research with salmonids and other fishes. Future studies using this method will be able to examine (1) relative ejaculate expenditure between territorial and sneaker males, (2) male ejaculate expenditure in relation to female characteristics (*e.g.* body size and genetic compatibility) and (3) the effect of successive spawnings on sperm depletion both over short (days) and long (the spawning season) time periods.

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