

Fertilization mode, sperm characteristics, mate choice and parental care patterns in *Artedius* spp. (Cottidae)

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The reproductive biology of three species in the genus *Artedius* (*A. harringtoni*, *A. lateralis* and *A. fenestralis*) was studied, including parental behaviour, mate choice, fertilization mode, gamete morphology and behaviour, and gonad morphology to help understand the coevolution of reproductive characteristics with potentially different modes of fertilization. In all three species, males guard multiple clutches at oviposition sites. In laboratory mate-choice experiments, males appeared to prefer to defend sites containing eggs and were better able to attract mates at these sites. There was evidence that internal gamete association, although more pronounced in *A. harringtoni*, existed in all three species. Sperm behaviour and morphology and histological analysis of gonads suggested that *A. fenestralis* and *A. lateralis* were more likely to engage in typical external fertilization. The adoption of eggs and their effect on female mate-choice patterns appeared to reduce the importance of certainty of paternity in these species, and probably increased the evolutionary stability of male parental care.

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Key words: external fertilization; internal gamete association; mate choice; paternal care; sexual selection; sperm competition.

INTRODUCTION

A change in fertilization mode, with shifts between external and internal fertilization, has occurred multiple times within the bony fishes (Wourms, 1981; Goodwin *et al.*, 2002; Reynolds *et al.*, 2002). This change, especially from external to internal fertilization, with subsequent development of embryos within the female, should involve a suite of adaptations, including the evolution of intromittent organs and sperm capable of fertilizing eggs in ovarian fluid in males, and the evolution of reproductive organs able to supply nutrients and expel waste products from developing embryos in females.

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In one group of fishes, the cottids (Cottidae and related groups), copulation and internal fertilization appear to have become decoupled. In multiple lineages, copulation occurs, yet eggs are not fertilized internally. Instead, sperm enter the ovary, associate with the ovulated egg at the micropyle, but do not enter and fertilize the egg until they are oviposited (Munehara *et al.*, 1989). Thus, the potential constraints of embryo development in the female are avoided in cottids, and internal gamete association is widespread within this group of fishes (Ragland & Fischer, 1987; Munehara *et al.*, 1989, 1991; Koya *et al.*, 1993).

The examination of this fertilization pattern in cottids, termed internal gamete association (IGA) (Munehara *et al.*, 1989), is of potential interest to investigators from multiple disciplines. From the viewpoint of gamete and gonadal morphology and physiology, the evolution of copulation and internal fertilization involves several adaptations for both males and females. Sperm morphology in fishes is consistently different among species with different fertilization modes, with internal fertilizers having longer sperm than external fertilizers (Stockley *et al.*, 1996). This pattern also exists in at least one invertebrate taxon (Raff *et al.*, 1990). Sperm in fish species with internal fertilization also tend to have elongated sperm heads that are either pointed or flattened (Jamieson & Leung, 1991; Ishijima *et al.*, 1998). An additional difference in sperm between species with external fertilization and internal gamete association is the range of osmolalities in which sperm are active. Species with IGA produce sperm that are active at the low osmolalities (*c.* one third that of sea water) that approximate internal body fluids, but are not motile in sea water (Koya *et al.*, 1993). In contrast, cottids with external fertilization have sperm that are active in sea water and at much lower osmolalities, and at least in one species sperm are active at an osmolality comparable to female ovarian fluid (Hayakawa & Munehara, 1998, 2002). There is also some evidence that the ovarian fluid of females in species with IGA needs to differ from serum levels in order to prohibit sperm from fertilizing the egg and inducing development in the ovary (Koya *et al.*, 1993; Munehara *et al.*, 1994a, 1997; Hayakawa & Munehara, 1998).

In fishes, neither internal fertilization nor IGA is typically associated with paternal care (Gross & Shine, 1981; Clutton-Brock, 1991). With the exception of seahorses and pipefishes, in which males carry eggs and females have the copulatory organs, this combination of copulation and paternal care is unique to the cottids and one non-cottid, *Pantodon buchholzi* Peters (Breder & Rosen, 1966; Clutton-Brock, 1991), within fishes. One previously reported case of internal fertilization and paternal care in the cardinalfish *Apogon imberbis* (L.) (Garnaud, 1962), now appears to be a case of external fertilization (M. Rasotto, pers. comm.). Ragland & Fischer (1987) proposed two potential hypotheses for the combination of IGA and paternal care: first, that females stay near males and that paternity remains high, thus avoiding high levels of cuckoldry, and second, that eggs serve as courtship devices (first hypothesized by Rohwer, 1978), with males defending eggs and oviposition sites to obtain copulations with females. Munehara *et al.* (1994b) found support for the latter, but not the former, hypothesis in *Alcichthys alcicornis* (Herzenstein), with males commonly providing care to clutches that they had not fathered. The ability of males to continue to obtain matings at a spawning site, and the role of eggs in attracting additional mates has been suggested as an important component of selection pressure for

the evolution of paternal care in fishes (Barlow, 1964; Williams, 1975; Blumer, 1979; Gross & Shine, 1981; Gross & Sargent, 1985; Clutton-Brock, 1991; Jamieson, 1995; Petersen, 1995). Examining male care in species where it is potentially decoupled from paternity could provide additional insight into the evolution of paternal care in fishes.

For many fishes, changes in morphology, with the male or the female developing an intromittent organ, is the most common external indicator of fertilization mode. In the cottid genus *Arteidius*, there is extreme variation in male genitalia, with one species, *Arteidius harringtoni* (Starks), having a large penis, while sympatric congeners in the cold-temperate eastern Pacific, *Arteidius fenestralis* Jordan and Gilbert and *Arteidius lateralis* (Girard), do not have an obvious intromittent organ and have been assumed to have external fertilization (Froese & Pauly, 2004). *Arteidius harringtoni* appears to have internal gamete association (Ragland & Fischer, 1987); in addition, there are two incidental reports in the literature suggesting that *A. lateralis* also has some form of internal gamete association or internal fertilization (Hubbs, 1966; Ragland, 1987). Some cottid species without copulatory organs are known to be able to inseminate females: *Blepsias cirrhosus* (Pallas) (Munehara *et al.*, 1991) and *Hemitripterus villosus* (Pallas) (Munehara, 1996).

In this study, the reproductive biology of these three sympatric *Arteidius* species was examined. External male morphology, gonadal structure, fertilization mode, sperm longevity and behaviour, and patterns of mate choice and parental care were examined to evaluate differences among three species in this genus. The initial goal of the study was to determine what, if any, differences existed between a species with internal gamete association and closely related species with external fertilization.

MATERIALS AND METHODS

FIELD OBSERVATIONS

Observations were made intertidally and subtidally in the area surrounding San Juan Island, Washington, U.S.A. from December to March in 1997–1998 and 2000–2001. When fishes were seen guarding eggs, the individuals were collected to determine the sex of the guarding parent or notes were taken on colouration and morphology of the guarding individual. Oviposition sites were also tagged in the intertidal at Mitchell Bay, San Juan Island (48°34' N; 123°10' W) to determine the occurrence and activity of individuals at oviposition sites.

For *A. harringtoni*, observations were combined with those from Ragland & Fischer (1987). For the other two species, there is a published observation of male parental care in *A. fenestralis* (DeMartini & Patten, 1979), and there are no reliable published accounts of adult parental behaviour in *A. lateralis*.

FERTILIZATION MODE

The fertilization mode in *Arteidius* spp. was checked in two ways: 1) ovaries were examined histologically for signs of sperm. Sperm have been observed in other cottids with internal gamete association (Koya *et al.*, 1994, 2002) using similar preservation and staining techniques; 2) a simple laboratory experiment was performed to test for internal gamete association. Gravid females were squeezed to remove some eggs, and

approximately half the clutch was fertilized with sperm squeezed from two males, while leaving the other half to develop without artificially added sperm. Development of the clutch with sperm added tested for the viability of the eggs, while development of both clutches would indicate that sperm were already present in the ovary with the eggs (or less likely, that asexual reproduction was occurring). Development of neither clutch would suggest that the squeezed eggs were not viable. Previous work by Ragland & Fischer (1987) showed that eggs taken from *A. harringtoni* develop without the addition of sperm, supporting the hypothesis of this species having internal gamete association. Neither of the other two species had been previously tested.

GONAD HISTOLOGY

Histological analyses were performed on fresh samples for all three species and supplemented with museum samples from the University of Washington fish collection (*A. fenestralis*: Cat. # 3038; *A. lateralis*: Cat. # 025807, # 17412, # 00903201457, # 17430). The whole reproductive apparatus of males and females was carefully excised and, in the case of museum samples, kept in 70% ethanol or, for fresh samples, fixed in Dietrich solution (900 ml distilled water, 450 ml 95% ethanol, 150 ml 40% formaldehyde, 30 ml acetic acid), dehydrated in ethanol, embedded in Paraplast, sectioned serially at 7 µm and mounted on slides.

Slides were stained with haematoxylin and eosin. The occurrence of polysaccharides was examined with the reaction of periodic acid Schiff (PAS) (Pearse, 1985). Alcian blue was used to distinguish the nature of any polysaccharides present: alcian blue at pH 1 and pH 2.5 respectively, to detect sulphated and non-sulphated mucins (Pearse, 1985). Proteins were stained with the mercury bromophenol blue method (Pearse, 1985). A total of six males [standard length, L_S , 80.6 ± 10.5 mm (mean \pm s.d.), range: 71.3–92 mm] and four females (66.1 ± 3.1 mm, range: 61.9–69.5 mm L_S) of *A. harringtoni* 10 males (83.5 ± 10.9 mm, range: 64–98.5 mm L_S) and 20 females (75.9 ± 10.4 mm, range: 57.2–101.3 mm L_S) of *A. fenestralis* and 10 males (93.6 ± 10.6 mm, range: 75.7–115 mm L_S) and 10 females (80.7 ± 13.7 mm, range: 62.3–101.3 mm L_S) of *A. lateralis* were analysed.

SPERM MORPHOLOGY AND BEHAVIOUR

Gross sperm morphology was measured using light microscopy. For five males of each species, sperm were obtained by gently applying pressure to the male's abdomen, and once motility had ceased sperm dimensions were measured directly from a monitor attached to a video camera attached to the compound microscope. For each of 10 sperm, the total length of the sperm and the length of the head (head plus any visible midpiece) were measured and converted to µm. For statistical analysis, individual was nested within species, and for tests showing significant effects Tukey's *post-hoc* test was performed to determine specific differences among species.

Sperm motility was measured at two salinities, full-strength sea water (salinity *c.* 27–30 in Friday Harbor during the winter) and one-third strength sea water (diluted with deionized water). One-third strength of sea water has the approximate osmolality of the fish, and presumably of the ovarian fluid of the female (Koya *et al.*, 1993). Sperm were extruded from males and randomly placed on four depression slides with water already present, two at each salinity. One slide at each salinity was observed on the microscope with continuous video recording until sperm forward movement ceased. It was confirmed that sperm motility was not truncated by the higher temperature of the stage by keeping the replicate slide at ambient water temperature (*c.* 10° C) on a shelf on a seawater table and checking it for motility after sperm ceased moving in the first slide. Sample sizes were five males for *A. harringtoni* and *A. fenestralis*, but only two for *A. lateralis*. In addition, sperm from two males of *Oligocottus maculosus* Girard, a species known to have IGA (DeMartini & Patten, 1979; C. Petersen, pers. obs.), were also observed for comparison with *Artedius* spp.

MATE CHOICE EXPERIMENTS

Mate choice experiments were done with *A. fenestralis* and *A. harringtoni* (there were too few *A. lateralis* individuals to conduct laboratory experiments).

Two males were placed in the centre of a large, shallow aquarium (sea water table, 0.6 by 1.2 m). Once males had established themselves at sites placed at either end of the tank (empty tests of the giant acorn barnacle *Balanus nubilus* for *A. harringtoni*, ceramic tiles for *A. fenestralis*), a female was added to the middle of the tank. The oviposition sites were monitored at least daily, and the presence of any egg masses was noted.

To examine the effects of eggs on mate choice patterns, two types of experiments were conducted. In the first type of experiment, a male with eggs was placed together with a male with an oviposition site but no eggs. A female was added to the tank, and subsequent oviposition was noted. In the second type of experiment, two males were added to a tank, where one of the two oviposition sites contained a clutch that neither male had seen before. Which site each male took was noted, and the next day a female was added to the tank and any subsequent oviposition was noted. Clutches that were in the first half of development (<1 week) were used in these experiments. Estimates of egg development time for *A. harringtoni* at laboratory water temperatures (*c.* 10° C) is *c.* 2 weeks (Ragland, 1987).

For data analysis, when there were no differences between species results were combined. The effects of male size, the side of the tank where oviposition occurred, the oviposition site for *A. harringtoni* (individual barnacle tests) and the presence of eggs on patterns of female oviposition were examined. Statistical analyses on mate-choice experiments were performed assuming the trials were independent events, despite the fact that some individuals were used multiple times in experiments. This was most problematic for the role of male size in the mate-choice experiments, since the males at one extreme in size range could count multiply for the same directional trend, but the result might be caused by a trait that covaried with size in these individuals. This is the most common way these results are reported in the literature, however, although there is increased potential for a type I statistical error.

RESULTS

FIELD OBSERVATIONS

In all three species males were observed at oviposition sites with eggs. In *A. lateralis*, eggs were found in the low intertidal [*c.* 0.3 m mean lower low water (MLLW) and below]. When sites were either slightly underwater or just exposed, males were often found attending spawns. If sites were substantially above the water line, no adults were present, although, when checked later when less exposed to air males were often found at these sites. Only males were found at oviposition sites.

In *A. fenestralis*, clutches were observed both intertidally and subtidally. In both cases males were found guarding multiple clutches. In one case, the male was captured, and within 10 min several large pandalid shrimp were eating the eggs at the oviposition site. In *A. harringtoni*, earlier reports by Ragland & Fisher (1987) that male guarding occurs in this species were confirmed, and several males that were at sites with eggs were collected.

SEXUAL DIMORPHISM

In all three species, males were larger than females, although the degree of difference varied considerably among species (Table I). In both *A. harringtoni*

TABLE I. Reproductive characteristics of the three *Arteidius* species

Species Characteristic	<i>Arteidius harringtoni</i>	<i>Arteidius fenestralis</i>	<i>Arteidius lateralis</i>
Sexual size dimorphism			
Mean \pm s.d. male L_S (mm) (<i>n</i>)	75 \pm 11 (99)	84.2 \pm 8.0 (13)	91.5 \pm 9.0 (10)
Mean \pm s.d. female L_S (mm) (<i>n</i>)	55 \pm 6 (47)	75.1 \pm 13.0 (17)	82.4 \pm 18.0 (8)
Size ratio (M : F)	1.36	1.12	1.11
Source	Ragland & Fischer (1987)	UW collection #3038	1998 and 2001 field collections
Male genital papilla			
Length (mm) (<i>n</i>)	11.9 (11)	1.93 (6)	1.85 (2)
Per cent of L_S	15.8	1.8	1.9
Mean \pm s.d. sperm head length (μ m)	5.24 \pm 0.62	3.48 \pm 0.44	3.30 \pm 0.40
Mean \pm s.d. sperm total length (μ m)	40.0 \pm 2.7	34.7 \pm 1.1	30.3 \pm 2.2
Sperm motility in full sea water	Low or no motility in four of five males	Highly motile	Highly motile
Sperm motility in one third sea water	Highly motile	Highly motile	Highly motile

UW, University of Washington.

and *A. fenestralis* the ratio of male : female L_S was very high, with males averaging 25–45% longer than females depending on the collection used. In *A. lateralis*, males were longer in all of the collections, but only 3–32% (11% for the field collections at San Juan Island). In all three species, these large males also had colouration patterns that differed from those of females. The most distinct was *A. harringtoni*, where males had darker bodies, bright orange throats and had much larger orbital cirri than females (Ragland & Fischer, 1987). In *A. lateralis*, males tended to have more pink or red colouration around their head, and in *A. fenestralis* males had more colourful dorsal fins, with red to yellow colouring throughout most of the spinous dorsal fin, and two dark areas on the anterior and posterior margins of the fin.

The size of the male genital papilla differed dramatically among species: *A. fenestralis* and *A. lateralis* had very small genital papillae and *A. harringtoni* had a genital papilla averaging 16% of the male L_S (Table I).

GONAD HISTOLOGY

In all the three species of *Artedius*, the main testicular ducts and a spermatic duct were enlarged [Fig. 1(a)]. The testes were of the unrestricted spermatogonial type (Grier, 1981), with all the different stages of spermatogenesis present [Fig. 2(a)]. In *A. fenestralis* and *A. lateralis*, males had a very similar gonadal structure. The sperm transport system (main testicular ducts and spermatic duct) was multichambered and functioned as sperm storage organ [Fig. 2(b)]. Chamber inner walls were lined with a monolayered epithelium, and lumina appeared completely filled with sperm. A scant secretion, consisting of syalo and solfo-glycoproteins, was visible, mainly in the anterior portion of the main testicular ducts [Fig. 2(b)(inset 1)].

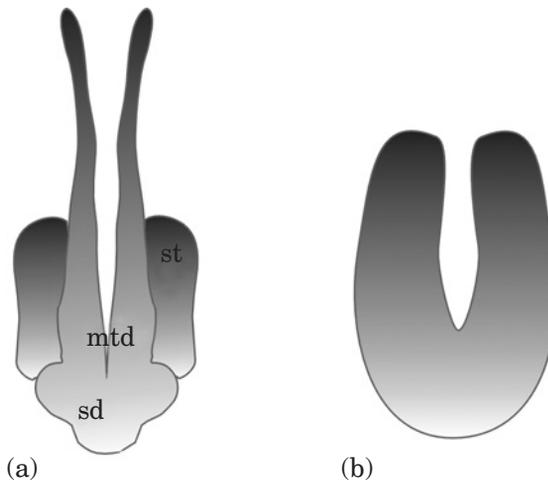


FIG. 1. Schematic drawing of the reproductive apparatus of the three species of *Artedius*: *Artedius fenestralis*, *Artedius lateralis* and *Artedius harringtoni*.: (a) male and (b) female. mtd, main testicular duct; sd, sperm duct; st, spermatogenic tissue.

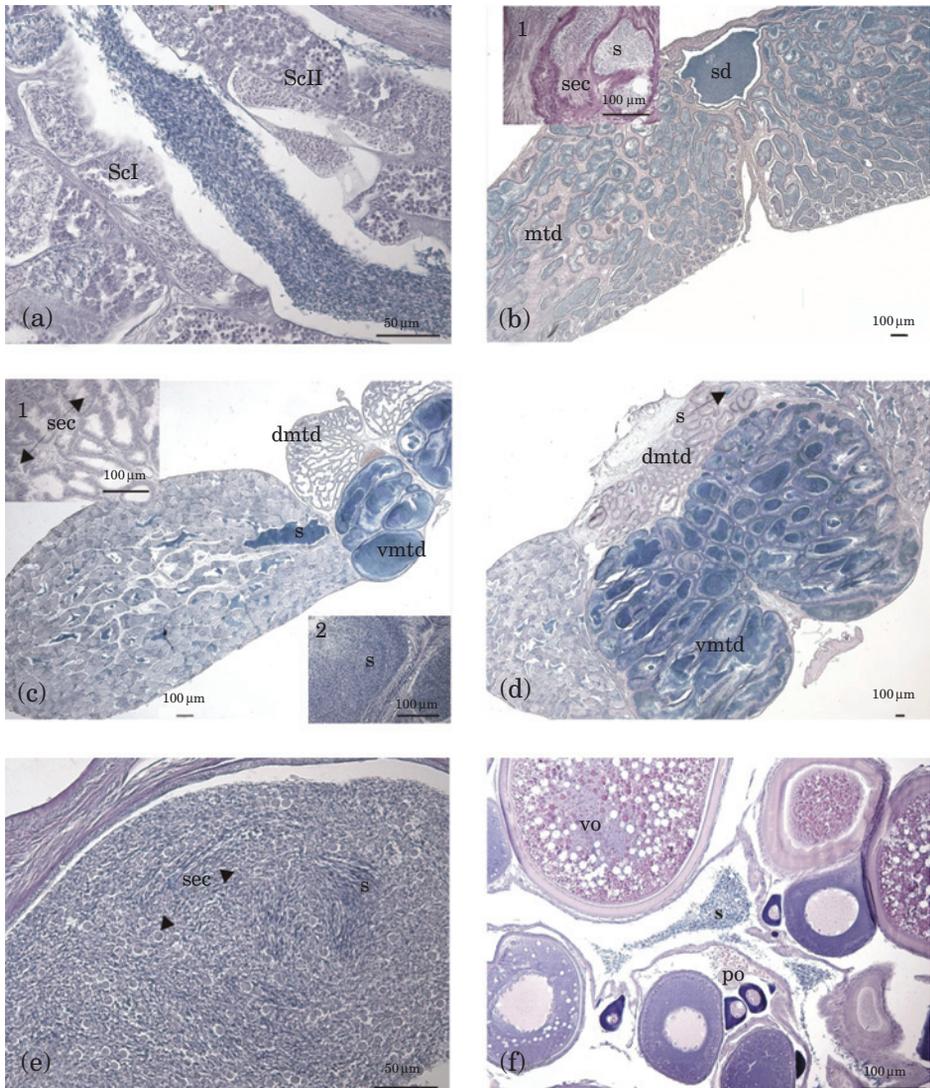


FIG. 2. (a) *Artedius harringtoni* male (78.5 mm L_S): testis presenting different stages of spermatogenesis. (b) *Artedius fenestralis* male (96.5 mm L_S): multichambered sperm transport system with lumina completely filled with sperm; (inset) the main testicular duct, chamber lumina present sperm and secretion. (c) *Artedius harringtoni* male (78.5 mm L_S): anterior section of the reproductive apparatus, main testicular ducts present a dorsal portion with small chamber with secretion (inset 1) and a ventral portion with large chambers filled with sperm (inset 2). (d) *Artedius harringtoni* male (92.0 mm L_S): posterior portion of the reproductive apparatus, the dorsal portion of the main testicular ducts present some chambers with sperm. (e) *Artedius harringtoni* male (92.0 mm L_S): section of the copulatory organ, sperm duct present sperm mixed with secretion. (f) *Artedius harringtoni* female (66.4 mm L_S): ovary presenting oocytes at different stages of development and sperm in the ovarian cavity, between lamellae. All sections are stained with haematoxylin and eosin. dmtd, dorsal main testicular duct; vmt, ventral main testicular duct; mtd, main testicular duct; po, previtellogenic oocytes; s, sperm; scI, primary spermatocytes; scII, secondary spermatocytes; sd, sperm duct; sec, secretion; st, spermatogenic tissue; vo, vitellogenic oocyte.

Males of *A. harringtoni* also had a multichambered sperm transport system, but in this species, the system showed two morphologically and functionally distinct parts: a ventral portion mainly storing sperm and a dorsal one secreting mucins [Fig. 2(c)]. The dorsal part had smaller chambers, lined with a thicker epithelium, than the ventral portion [Fig. 2(c) (inset 1)]. Chambers of both portions contained secretion, consisting of syalloglycoproteins, in their lumina. Those of the ventral part, however, contained only a small amount of secretion and were instead filled with sperm. Sperm appeared to be embedded in the secretion and at times formed small bundles in the chamber lumina with the sperm heads all oriented towards the middle of the sperm mass [Fig. 2(c) (inset 2)]. Towards the posterior end of the duct, sperm were increasingly observed embedded in the secretion, and were present also in the dorsal part of the duct [Fig. 2(d)]. In the duct of the copulatory organ, sperm appeared mixed with the secretion [Fig. 2(e)].

In all three species of *Artemius* the ovary was a hollow bilobed organ [Fig. 1(b)] that contained oocytes at different stages of development (asynchronous type, Nagahama, 1983) [Fig. 2(f)]. Sperm were observed in the ovarian cavity of two of four females in *A. harringtoni* [Fig. 2(f)]. No sperm were observed in the ovaries of either of the other two species (*A. lateralis*, $n = 10$; *A. fenestralis* $n = 20$). The difference in sperm presence in the ovary between *A. harringtoni* and the other two species is highly unlikely due to chance ($P = 0.011$, Fisher-exact test). All collections were from the reproductive season of these species.

SPERM MORPHOLOGY AND BEHAVIOUR

The sperm morphology of the three *Artemius* species was significantly different (Table I). *Artemius harringtoni* had the largest sperm, both in terms of head length and total length (nested ANOVAs, $P < 0.001$ for the effect of species in both cases, all four possible pair-wise comparisons with *A. harringtoni* $P < 0.001$). *Artemius fenestralis* was intermediate in total sperm length, differing significantly from both species (Tukey test, d.f. = 2, 134, $P < 0.001$), but was similar to *A. lateralis* in sperm head size (Tukey test, d.f. = 2, 134, $P = 0.55$).

Individuals also varied significantly in sperm characteristics within species in both ANOVAs (effect of individual nested within species, d.f. = 13, 134 $P < 0.001$ for both comparisons). Species was the dominant variable in the analysis, accounting for 79% of the total variation in sperm length and 75% of the total variation in sperm head length.

Sperm motility was very similar for both *A. fenestralis* and *A. lateralis*. In both species, sperm were very active both in full sea water and one third strength sea water. In *A. fenestralis* ($n = 5$ individuals), some sperm were active in both salinities to 12–15 min, with one case of activity to 60 min in sea water. In *A. lateralis* ($n = 2$ individuals), some sperm were active to 18 min in both salinities.

In contrast, only two of the five individuals of *A. harringtoni* had sperm that were initially active in sea water, with one active for <10 min, although all were active in one third strength. This pattern mirrored the pattern seen in the cottid with known internal gamete association, *O. maculosus*, where both individuals had inactive sperm in full strength sea water, but the sperm were highly active at one third strength sea water. In *A. harringtoni*, in two cases initially inactive sperm became slightly active after 12 min in full strength sea water.

FERTILIZATION MODE

In both *A. lateralis* and *A. fenestralis*, eggs extruded in the laboratory proceeded to develop without additional sperm, although this was an uncommon occurrence. In *A. fenestralis*, in one case all of the eggs developed in both the sperm added and the no-sperm added treatments, while in the other two cases only the eggs in the sperm-added treatment developed. In *A. lateralis*, in one case all of the eggs developed in both treatments, while in a second case, a few eggs developed to gastrulation in the sperm-added treatment, but most of the clutch in this treatment and all of the eggs in the no-sperm treatment failed to develop. Since the eggs in this second replicate failed to develop normally and stopped developing quite early, this case was not counted as evidence of external fertilization of this clutch. All of the other attempts, including all those with *A. harringtoni*, showed no signs of development in either treatment, with or without the addition of sperm. One case of successful development of a clutch laid in the laboratory by a female *A. harringtoni* in the absence of males, however, was observed.

MATE CHOICE

In both the species examined, *A. fenestralis* and *A. harringtoni*, larger males tended to obtain clutches at a higher rate when eggs were not initially present. In 16 of 22 cases the larger male received eggs (10 of 15 in *A. harringtoni*, six of seven in *A. fenestralis*; combined data, χ^2 , d.f. = 1, $P < 0.033$). There was no female preference for which side of the tank eggs were deposited on, and the identity of the oviposition site used also had no apparent effect on mate choice.

In *A. fenestralis*, females also preferred to mate with males already caring for eggs. When one male defending a clutch was used in the mate choice experiment, that male received another clutch in all four replicates in *A. fenestralis* where additional females oviposited. In two cases the smaller male had eggs, and was able to maintain ownership of the oviposition site and obtain additional eggs. When a single clutch was present and two males were added to the tank, the larger individual adopted the site with eggs all four times and in every case was the one to receive additional eggs.

The effect of current clutches on mate choice in *A. harringtoni* were in the same direction but not as strong as in *A. fenestralis*. Larger males tended to guard eggs, and in most cases received additional eggs. Larger males received additional eggs in six of eight cases where they had eggs, but also acquired an additional clutch in two of three cases where the smaller male had the clutch. In addition, in one case the larger male displaced the smaller male tending eggs, and the male with the newly adopted clutch received an additional clutch. In seven cases where two males were added to a tank when one site contained at least one clutch, the larger male adopted the site with eggs in all seven cases and received additional clutches all seven times.

Clutches sometimes showed signs of partial cannibalism, but the effect of partial clutch loss on female choice was unclear. In *A. harringtoni*, there were three instances where current clutches showed signs of partial clutch

cannibalism; in two cases that male received another clutch, while in the other case the male without eggs received the next clutch.

DISCUSSION

FERTILIZATION MODE IN *ARTEDIUS*

Among these three species in the genus *Arteidius*, there are both similarities and several differences among the species in their reproductive biology. All evidence, both from this and a previous study (Ragland & Fischer, 1987) support the hypothesis that *A. harringtoni* has internal gamete association. Sperm is found in the ovaries of females, eggs develop when artificially extruded from females in the absence of additional sperm, and males have a large penis. Eggs, however, were never observed to begin development until after they had been released by the female, so fertilization still appears to be external, as occurs in other cottids with IGA (Munehara *et al.*, 1989, 1991).

The case for internal gamete association in the other two species of *Arteidius* examined here is less clear. Single clutches of both species developed without sperm addition (*A. fenestralis*, this study; *A. lateralis*, Hubbs, 1966; Ragland, 1987; this study), strongly suggesting that sperm are able to enter and remain in the female ovary or oviduct. It thus appears that both *A. lateralis* and *A. fenestralis* are capable of internal gamete association. In some cases in *A. fenestralis*, however, development only occurred when sperm was artificially added to eggs, implying that for this species and probably for the similar *A. lateralis*, external fertilization without IGA can occur in nature.

There are several differences in sexual morphology and behaviour that suggest IGA may occur at a higher frequency in *A. harringtoni* than in the other two *Arteidius* species. The lack of a large male genital papilla in *A. lateralis* and *A. fenestralis* and the absence of obvious sperm in histological sections of ovaries of these two species, despite the high number of analysed females, may represent either a smaller number of sperm transferred in the two species without an obvious penis, or a lower rate of copulations in *A. lateralis* and *A. fenestralis*. That, combined with the mixed results in the fertilization mode experiment for *A. fenestralis* suggests that female *A. fenestralis* and *A. lateralis* may produce eggs or clutches that are fertilized both *via* IGA and the more common mode of external fertilization *via* sperm in the water column. This conclusion is reinforced by the data from the behaviour and morphology of sperm: the sperm of *A. lateralis* and *A. fenestralis* are closer in morphology to species with external fertilization than that of *A. harringtoni*, and the sperm were active both in sea water and in osmolalities similar to body fluids. This contrasts with species that appear to have obligate or near obligate IGA, where sperm show reduced or no motility in sea water (Koya *et al.*, 1993). Sperm motility in *A. harringtoni* appears very similar to sperm motility in *A. alpicornis*, with highest motility in osmolalities close to internal body fluids, and very limited activity in sea water (*c.* 3 min in *A. alpicornis*) (Koya *et al.*, 1993). There are also differences in the morphology of the sperm transport system among the three species, with *A. harringtoni* having the more abundant secretion associated with the ducts and sperm enveloped in this secretion with heads oriented towards the core of the sperm bundle.

The formation of sperm masses embedded in secretion is commonly observed in some internal fertilizers such as sailfin molly *Poecilia latipinna* (Lesueur) (Grier, 1975), halfbeaks *Zenarchopterus* spp. (Grier & Collette, 1987; Downing & Burns, 1995) and glandulo-caudine fishes (Burns *et al.*, 1995). In *Artedius* species, however, the function of the ducts and their relationship with internal gamete association *v.* external fertilization has not yet been studied. All of these differences among these congeners suggest functional differences in fertilization within this genus.

Artedius fenestralis is not the first species of cottid where two modes of fertilization have been proposed. Munehara (1988) proposed that in *A. alciornis*, IGA was the rule, but that the first clutch of the year was fertilized externally by sperm observed leaking from the female during and after copulation immediately post-spawning. This is because copulation was only observed immediately after oviposition, leaving the question of how the earliest clutch was fertilized. Although this mechanism may seem surprising, excess milt pouring out of the female genital duct post-copulation, while the female is closely associated with the eggs, has also been reported for *Clinocottus recalvus* (Greeley) (Morris, 1952). In *Artedius* spp., external fertilization of a clutch might occur throughout the season, and external fertilization cannot be excluded for *A. harringtoni*, which may have a similar reproductive biology to *A. alciornis*.

Copulation near the time of spawning has been observed in other sculpin species with IGA and no parental care, such as *O. maculosus* (C. Petersen, pers. obs.) and *C. recalvus* (Morris, 1952), although some other species appear to copulate 'in an atmosphere of carefree promiscuity' (*Oligocottus snyderi* Greeley, Morris, 1956). It is possible that copulation is more likely to be successful after oviposition, when the female genital duct is distended. In the laboratory, males are much more attentive to female *O. maculosus* immediately before spawning, and appear to use spawning as a cue for courtship and copulatory behaviour (C. Petersen, pers. obs.). Although copulation was not observed in *Artedius* spp., behaviours implying copulation were observed in *A. harringtoni* while both individuals were inside a barnacle oviposition site either during or immediately after oviposition.

MATE CHOICE AND PATERNAL CARE IN *ARTEDIUS*

In all three species, males provided parental care at oviposition sites. Given the presence of sperm in ovaries of *A. harringtoni* and reports of eggs developing in the absence of a male or sperm at oviposition for all three species, all species appear to have some degree of IGA, although it may vary among the species. This pattern of male parental care and internal fertilization (or at least internal gamete association) is very unusual in fishes (Clutton-Brock, 1991), although it appears to have evolved multiple times within the cottids. The phylogeny of the cottids is not well resolved, but the most recent phylogenetic hypothesis places *Alichthys* and *Artedius* in very different parts of the phylogeny (Yabe, 1985).

The high frequency of paternal care has been a focus of much of the theoretical and review literature on the evolution of parental care in fishes (Perrone & Zaret, 1979; Gross & Shine, 1981; Blumer, 1982; Gross & Sargent, 1985;

Clutton-Brock, 1991). Although several hypotheses exist to explain this association, the predominant hypothesis is that male costs of parental care are lower than female costs, either because they can attract additional mates by staying at sites or because they have lower relative future reproductive value compared to females (Sargent & Gross, 1986). The presence of clutches at a site enhances the benefits in terms of egg survivorship associated with the site, either through predator dilution (Rohwer, 1978) or through increased male defence of the site (Sargent & Gross, 1986). These clutches thus become courtship devices, and either have no effect or increase the mating success of males at these sites (Jamieson, 1995). Female preference for eggs in a nest has been hypothesized to have led to the adoption of unrelated eggs as part of a male mating strategy (Rohwer, 1978; Ridley & Rechten, 1981; Marconato & Bisazza, 1986; Unger & Sargent, 1988). This pattern has only been reported in fish species with external fertilization, but the mate-choice experiments suggest that eggs are important courtship devices in *Artedius* spp. and that males are very willing to adopt unrelated eggs. The hypothesis that males willing to guard young become preferred mates for gravid females and enjoy greater mating success than males unable or unwilling to guard has recently been proposed to account for cases of exclusive paternal care in arthropods (Tallamy, 2000, 2001) in addition to its proposed importance throughout the fish paternal care literature (Clutton-Brock, 1991; Jamieson, 1995; Petersen, 1995).

The presence of male parental care in species with IGA and without obvious ways to maintain high paternity of clutches suggests that the benefits for staying at oviposition sites may be related to copulations as well as increasing survivorship of clutches. An association between spawning and copulation would strengthen the association of males with females during oviposition, and if there are preferred oviposition sites, would increase the benefits for males defending oviposition sites. Thus, the suspected lower certainty of paternity in these species may not preclude male care, especially since defending an oviposition site appears likely to increase copulatory rate in at least some species with IGA.

There is evidence from the other species with IGA and paternal care that males care for clutches that they do not fertilize. Evidence relying on DNA fingerprinting has shown that in nature, males do care for unrelated embryos (Munehara *et al.*, 1994b). In fact, in the latter part of the breeding season, the majority of the eggs in a male's care were unrelated to him. Similar molecular paternity analysis of clutches in *Artedius* spp. should shed more light on the dynamics of mate choice and paternal care in this species.

Why this pattern of internal gamete association has not evolved into viviparity in this clade may be a unique result within fishes, and the association of copulation with paternal care in fishes challenges many of the ideas surrounding the importance of certainty of paternity and the evolution of paternal care in this group.

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