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Source: Journal of Vertebrate Biology, 72(22069)

Published By: Institute of Vertebrate Biology, Czech Academy of Sciences

URL: <https://doi.org/10.25225/jvb.22069>

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Male position in a sexual network reflects mating role and body size

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► Received 11 December 2022; Accepted 10 March 2023; Published online 6 April 2023

Abstract. Several species are characterised by male mating polymorphisms, which are often associated with sperm competition for which some phenotypes show specialisation. With high-resolution behavioural data, we used network analysis to quantify sperm competition intensity; the probability of overlap of the ejaculates of different males among competing male European bitterling (*Rhodeus amarus*) in a large experimental mesocosm. Implementing Bayesian inference with informative priors, we modelled sperm competition intensity among males adopting two alternative mating roles to understand how sperm competition intensity varied between roles as a function of body size. We demonstrated that larger males performing a “guarder” role experienced lower sperm competition intensity than smaller males. In contrast, for “sneaker males”, larger males experienced greater sperm competition intensity. We discuss these findings in the context of mating system evolution and the reproductive biology of bitterling.

Key words: Bayes’ theorem, mating tactic, posterior distribution, network analysis, sperm competition

Introduction

Selection that acts on differences in fitness among individuals due to the number and identity of their mates is termed sexual selection (Andersson 1994). Sexual selection tends to act more strongly on males, a consequence of males producing numerous small gametes (spermatozoa), while females, by definition, produce a relatively smaller number of large gametes (eggs). An outcome is that males typically experience higher pre-copulatory sexual selection.

Alternative mating phenotypes characterise males of many animal species. Two male mating phenotypes are often evident; one is characteristically large-bodied, territorial, aggressive and colourful, actively

courts females, drives away rivals and is usually termed a “guarder” phenotype. Other males in the same population expressing an alternative phenotype may be small, cryptic and frequently reproduce by attempting to fertilise the eggs of females they have not courted through “sneaky” fertilisations. This guarder-sneaker distinction has been described across a range of vertebrate and invertebrate species (Taborsky 1998).

The mechanism by which alternative mating phenotypes are maintained in the same population is probably through negative frequency-dependent selection; as the frequency of one male mating type increases, its fitness declines relative to the other, while the rarer phenotype experiences elevated

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fitness. Because sexual selection on traits closely linked to fitness is usually strong (Kokko & Jennions 2014), changes in male mating phenotype frequencies are likely to be rapid. At a population level, different mating phenotypes are predicted to generate equivalent fitness payoffs (Shuster & Wade 2013). The terms reproductive “strategy” and “tactics” are frequently used in the context of male mating polymorphisms and are not synonyms (Wootton 1984). For a given reproductive strategy, which is largely fixed, there is a range of mating tactics that reflect changes to behaviour, morphology and physiology that are nested within the constraints imposed by the broader strategy.

Sperm competition occurs when the spermatozoa of two or more males compete to fertilise the same eggs (Parker 1998). The “risk” of sperm competition is the probability that a male’s sperm will compete with the sperm of other males. The “intensity” of sperm competition is the extent of overlap of the ejaculates of different males. It is a function of the number of males that engage in sperm competition, as well as the quantity of sperm they contribute to a mating. Alternative male mating phenotypes are commonly associated with an elevated risk of sperm competition, particularly for the sneaker phenotype, because they usually mate in the presence of at least one competitor, typically a guarder male, and often other sneakers. An outcome is that sneakers are predicted to show adaptations for sperm competition, such as relatively (or even absolutely) larger testis size, larger ejaculates, and faster swimming spermatozoa (Montgomerie & Fitzpatrick 2019).

Across species, the prediction is that with increased competition among the ejaculates of rival males, selection will tend to increase investment in sperm competition if the sperm from different males has an equal probability of fertilising female’s eggs, a state often referred to as a “fair raffle” (Parker 1998). An outcome of a fair raffle is that those males that release larger or more frequent ejaculates will fertilise a larger share of eggs, with the consequence that sperm competition will select for greater sperm production.

This study used European bitterling (*Rhodeus amarus*) to explore the relationship between sperm competition intensity and male mating role. Bitterling (Cyprinidae, Acheilognathinae) are small cyprinid fishes typically found in freshwater lakes and rivers across Europe and East Asia (Duyvené de Wit 1955, Wiepkema 1961, Smith et al. 2004). They have an unusual mode of reproduction, with females

depositing their eggs in the gill cavity of freshwater bivalve molluscs (hereafter “mussels”) using a specialised ovipositor (Dyková & Reichard 2023), where they are fertilised by the male and complete development (Boeseman et al. 1938, Duyvené de Wit 1955). This unusual mating system has led to the evolution of striking reproductive behaviour and physiology, as well as unusual egg morphology and larval development (Kawamura & Uehara 2005). The adaptations shown by bitterling to this mode of reproduction offer an unusually tractable model system for understanding the factors that underpin mating system evolution.

Ostensibly the mating system of bitterling, at least the species examined in detail, can be classified as resource-defence polygyny (Smith et al. 2004). Males express territoriality, and females are choosy, both of the sites of oviposition and mates. In addition, males perform alternative mating tactics and engage in pre-oviposition ejaculation, with males releasing spermatozoa over mussels before females deposit their eggs. Female European bitterling are also polyandrous, depositing multiple clutches of 3-5 eggs in mussels over a 6-8 week breeding season with multiple males, sometimes spawning with a single male but potentially with multiple males participating in a single spawning event (Smith et al. 2004). Females may also delay oviposition and engage in conspicuous spawning-related behaviours that encourage participation in spawning by multiple males (Smith & Reichard 2005).

Network analysis has been applied to animal social structure to measure sociality and group structure (Krause et al. 2015). Interactions between individuals in a population are not random; not all individuals interact with equal likelihood, and there can be striking differences in the number of social partners among individuals (Krause & Ruxton 2002). Network analysis can be applied as a tool to understand evolutionary questions, including reproductive dynamics “sexual networks” whereby nodes in a network represent individual males/females, with connections between them some form of interaction with a reproductive context (McDonald et al. 2013).

The application of network analysis to describe mating systems offers a powerful approach to understanding the operation of sexual selection and mating system evolution (McDonald & Pizzari 2016). “Sexual networks” are superior to standard quantitative approaches, which often cannot adequately capture the complexity of mating interactions (reviewed by



McDonald et al. 2013). A network-based approach also explicitly links local individual-level processes with population-level processes. This approach has been viewed as a key goal in ecology (Łomnicki 1988, Smith et al. 2000). Network analysis requires fine-grained data at the individual level but can potentially yield profound insights into mating systems, their flexibility, and the degree to which they can drive population-level processes. The development of new technological tools for recording interactions among individuals will potentially facilitate data collection of sufficient quality to generate sexual networks (McDonald et al. 2013).

This study used network analysis to quantify sperm competition intensity among competing male bitterling in a large mesocosm. Using Bayesian inference, we modelled sperm competition intensity among males adopting alternative mating roles to understand how sperm competition intensity varied as a function of body size. We predicted that larger males that performed a guarder role would experience lower sperm competition intensity than smaller males since they are more effective in guarding mating resources (freshwater mussels). In contrast, body size in sneaker males was predicted to be positively associated with sperm competition intensity since larger sneaking males would be more successful in participating in matings with rivals. Data for the study came from two separate experiments.

Bayesian inference is increasingly recognised as an essential tool for modelling ecological data. With Bayesian inference, we can directly address the probability that our hypothesis is true, conditional on the data; represented by the posterior distribution. An essential feature of the Bayesian approach is that probability is explicitly subjective and represents a measure of the degree of belief. The Bayesian posterior distribution is an expression of that degree of belief and is the product of the likelihood and prior information about the hypothesis that is independent of the data, which means that both data and existing information contribute to a probability estimate. Thus, while the more conventional frequentist approach tests a null hypothesis and assumes no relevant information irrespective of the number of times the same null hypothesis may have been examined previously, the more powerful Bayesian approach takes advantage of existing information to formulate the probability of whether a hypothesis is supported. Consequently, Bayes' theorem lends itself to an iterative exploration of probability.

Material and Methods

Experimental setup and behavioural observations

Two large-scale mesocosm experiments were conducted in an outdoor concrete pool at the Institute of Vertebrate Biology, Brno, Czech Republic, in May 2008 and 2022 using an identical protocol. The pool measured 12.4 × 6.0 m, and the water depth was 0.6 m. Water temperature varied naturally between 18 and 21 °C. The pool was stocked with 16 male and approximately 200 female *R. amarus*, captured by electrofishing in the River Kyjovka in the southeast of the Czech Republic. Fish were fed ad libitum daily with frozen chironomid larvae. Before stocking, males were individually marked with a pair of coloured elastomer tags placed in the dorsal musculature at the base of the dorsal fin; one mark on each side. Six colours of tags were used, thus enabling each male to be identified by a unique combination of colours. In addition, the male standard length (SL; from the tip of the snout to the origin of the caudal fin) was measured to the nearest 1 mm. Twelve *Unio tumidus* mussels were placed in sand-filled pots at numbered spawning sites spaced 2 m apart around three sides of the pool. One end of the pool was left free of mussels to let observers enter and leave the pool without disturbing experimental fish. Artificial plants (0.7 m length) were sited around the margins of the pool as refuges for fish.

After allowing the fish at least 48 h to settle in the pool and for males to establish territories around mussels, every spawning site was observed by a diver for 10 min twice each day; morning (09:00-12:00) and afternoon (12:00-15:00), in a randomised order. Observers recorded the timing of every sperm release by males over mussels and the identity of individual males based on their colour marks. Daily observations were repeated for four consecutive days in both years of the study (7 May-10 May 2008, 30 April-3 May 2022). An observation period of 10 min enabled multiple ejaculations while permitting every territory to be observed twice a single day.

Assigning male mating strategy

At the end of each study, all males were recaptured, killed in anaesthetic and measured (SL, nearest 1 mm). In 2022, males were also weighed (nearest 10 mg) and dissected to determine gonad mass (nearest 10 mg). In 2008, males were fixed in 4% formalin and stored in 70% ethanol. In 2022, males preserved in 2008 were remeasured, weighed (nearest 10 mg) and dissected to determine gonad mass (nearest 10 mg).

In 2022, one male proved to be an intersex individual, possessing testes of approximately 40% of the size predicted for its body size and containing ovarian tissue. This male was not observed to release sperm over mussels during the behaviour study and was excluded from subsequent data analyses.

Each male's gonado-somatic index (GSI) was calculated as: $100 \times \text{gonad mass} / \text{total body mass}$ (Wootton & Smith 2015). To categorise male mating roles as either "guarders" or "sneakers", a frequency distribution of male GSI was plotted for males in each year. The resulting bimodal GSI frequency distributions were decomposed to normal distributions using the *mix2normal* procedure in the *VGAM* package (Yee & Wild 1996) in R version 4.2.1 (R Core Team 2022). Individuals in the lower decomposed GSI distribution were categorised as guarders and upper distribution as sneakers.

Sperm network analysis

A network of male interactions based on overlapping ejaculations was built using the *igraph* package for network analysis in R (Csardi & Nepusz 2006). The probability of overlap in ejaculates was estimated for different males on the same day they were observed to release sperm over the same mussel during the same observation period. After ejaculation, the sperm drawn

into a mussel depletes through the filtering action of the mussel (Smith & Reichard 2013). We modelled sperm depletion using a half-normal distribution with standard deviation = 135 seconds, i.e. such that two ejaculates released 135 seconds apart would have a 67% probability of overlap. This function generated a distribution (Fig. 1) that matched empirical data for sperm depletion in mussels (Smith & Reichard 2013), enabling the generation of a sexual network based on overlapping ejaculates for the population of males in the experimental pool in each year of the study. To examine path lengths among males in the networks generated, we simulated 10,000 random networks with the same number of nodes and edges, calculated the average path length of the random networks and plotted their distribution. Finally, we compared this distribution with the average of the observed networks in 2008 and 2022.

Based on the probability of ejaculate overlap, the closeness centrality of males in each sperm network was estimated. Closeness centrality measures how close each node (i.e. male) in a network is to all other nodes. In the context of the present study, closeness centrality was taken as an index of the sperm competition intensity experienced by each male in the experimental mesocosm throughout the study in each year.

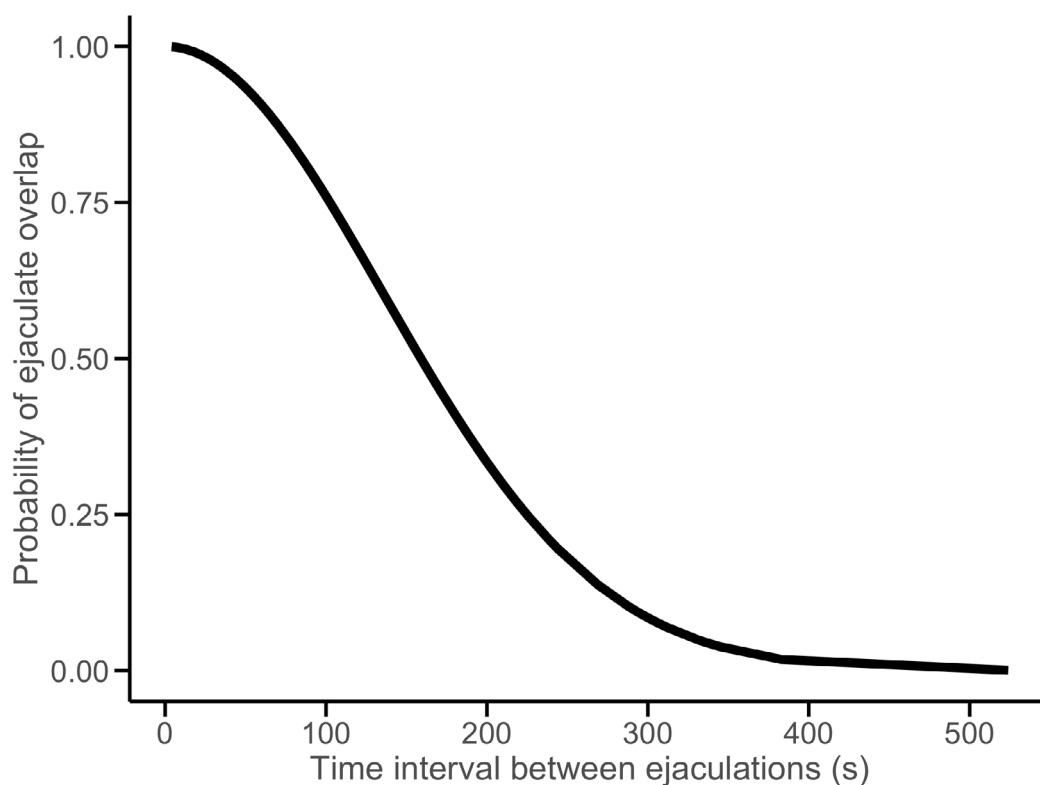


Fig. 1. Probability of overlap of the ejaculates of competing European bitterling males as a function of the time interval between ejaculations. The depletion of sperm over time arises from the clearance of sperm from the mussel gill cavity by filtration by the mussel (Smith & Reichard 2013).

Table 1. Mean (SD) of standard length (mm), mass (mg) and gonado-somatic index (% body weight) for male European bitterling used in the study in 2008 and 2022.

Variable	Mating tactic			
	Guarder		Sneaker	
	2008	2022	2008	2022
SL (mm)	57.2 (8.2)	51.4 (7.2)	58.0 (7.4)	45.3 (3.5)
Mass (mg)	4,077 (1356)	3,102 (1116)	3,978 (1365)	2,039 (540)
GSI	2.58 (0.35)	2.96 (0.41)	3.49 (0.31)	4.14 (0.30)

Modelling network closeness centrality

Despite following an identical protocol, data from 2008 and 2022 were not directly comparable; male body size distribution in 2008 was substantially greater than in 2022 (Table 1), which resulted in collinearity between year and SL, precluding the pooling of data. Consequently, we modelled closeness centrality for data collected in 2008 with a frequentist linear model that included male mating tactic (guarder or sneaker) and male SL, as well as the interaction between these variables. We subsequently modelled the closeness centrality of males in 2022 using a Bayesian linear

model, with informative priors based on model parameters from the frequentist linear model for 2008 data. This model also included male mating tactic (guarder or sneaker), male SL, and their interaction as parameters. We fitted the same model to data from 2022 with uninformative (default) priors.

The frequentist linear model was fitted using the *glmmTMB* package (Magnusson et al. 2017). Data exploration was conducted using the protocol of Ieno & Zuur (2015). After fitting, the model validation protocol of Zuur & Ieno (2016) was undertaken. Model

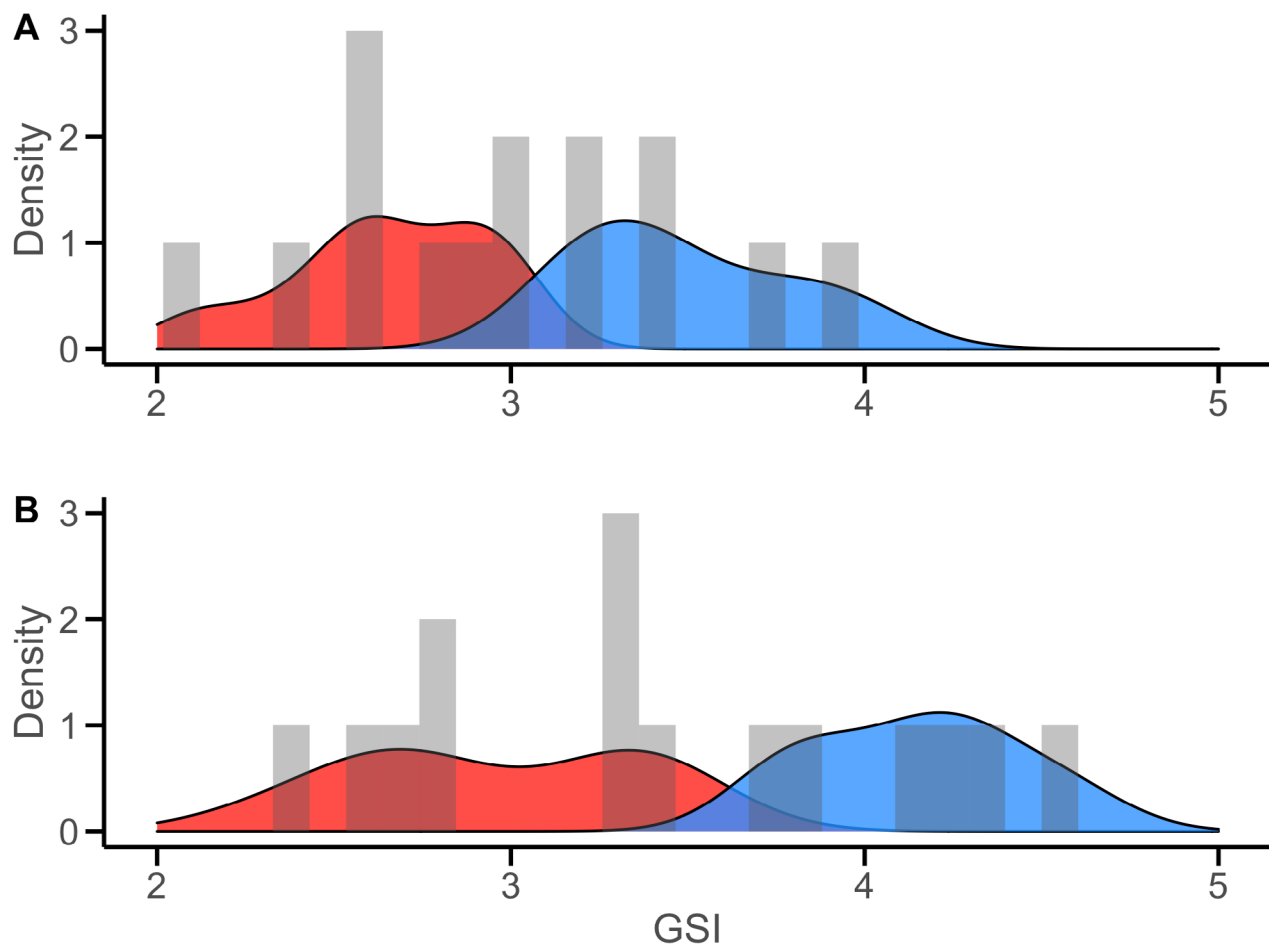


Fig. 2. Frequency density plot of the gonado-somatic index (GSI) of male European bitterling assigned to guarder and sneaker mating roles in 2008 and 2022. Gray bars indicate individual males.



probability integral transform (PIT) test (Fig. S5), and Bayesian residuals plots (Fig. S6).

Results

Assigning male mating role

GSI ranged from 1.95–3.96 in 2008 and 2.34–4.57 in 2022. In 2008, ten males were assigned to a guarder role and six to a sneaker role. In 2022, nine were assigned to a guarder role and six as sneakers. Summary data for males are shown in Table 1. Males were assigned to mating roles based on the distribution of their GSI, with discrete distributions in each year (Fig. 2, Table 1).

Table 2. Parameter estimates for frequentist Gaussian linear model fitted to network data from 2008, used as informative priors in the Bayesian linear model fitted to data from 2022.

Coefficient	Gaussian LM		
	Estimate	SD	P
Intercept	0.099	0.037	0.021
Role _(Sneak)	−0.107	0.067	0.137
SL	−0.0007	0.0006	0.320
Role _(Sneak) : SL	0.002	0.001	0.124

Network analysis

The timings of 223 ejaculations in 96 10-minute observation sessions were recorded in 2008, and 253 ejaculations in the same number of observation sessions in 2022. These data were used to generate a sperm network in each year (Fig. 3A, B) and calculate the closeness centrality of males in each network as an index of sperm competition intensity.

Modelling network closeness centrality

A frequentist linear model for data from 2008 showed a non-significant interaction between mating role and male length; the relationship between length and closeness centrality in guarders was negative, while in sneakers, it was positive (Table 2, Fig. 4). A Bayesian

linear model with informative priors, based on model parameter estimates for 2008, showed a statistically important relationship between male SL and closeness centrality for data from 2022 (Table 3, Fig. 5A). When this model was fitted with uninformative priors, there was no important relationship between SL and closeness centrality (Table 3, Fig. 5B). A sensitivity analysis showed the Bayesian linear model parameters to vary in proportion to the variation in the priors (Table S1, Fig. S7), indicating relatively high, but not excessive, model prior sensitivity. Reversing the analysis and instead fitting a Bayesian linear model for data from 2008, showed the same qualitative relationship between male SL and closeness centrality (Table S2). Checks also demonstrated the model to be robust. A cross-validated probability integral transform (PIT) test, which simulates data and evaluates the cumulative distribution function of predictions, showed a uniform distribution, indicating model adequacy (Fig. S5). Similarly, model residuals were homogenous and showed no marked patterns (Fig. S6).

Discussion

We used network analysis to quantify sexual network closeness centrality (a proxy for sperm competition intensity) among a small population of European bitterling in a large mesocosm. Using Bayesian inference, we modelled sperm competition intensity among individually-marked males performing alternative mating roles to understand how sperm competition intensity varied between mating roles as a function of body size. We predicted that larger males that performed a guarder role would experience lower sperm competition intensity than smaller males since body size correlates with dominance in bitterling (e.g. Casalini et al. 2009). Hence, larger males would be expected to more effectively exclude rivals from mating resources (freshwater mussels). In the case of a sneaker mating role, we predicted that body size would be positively associated with

Table 3. Posterior mean estimates for parameters of a Bayesian linear regression to predict the closeness centrality of male European bitterling as a function of male standard length (mm) and mating role modelled using a Gaussian LM fitted using Bayesian inference with INLA. CrI is the Bayesian 95% credible interval. The parameter σ is the standard deviation of the residuals.

Model parameter	Posterior mean	SD	Lower 95% CrI	Upper 95% CrI
Intercept	0.066	0.018	0.031	0.103
Role _(Sneak)	−0.085	0.034	−0.152	−0.020
SL	−0.0005	0.0004	−0.0012	0.0002
Role _(Sneak) : SL	0.0018	0.0007	0.0004	0.0032
σ	0.011	0.002	0.008	0.016

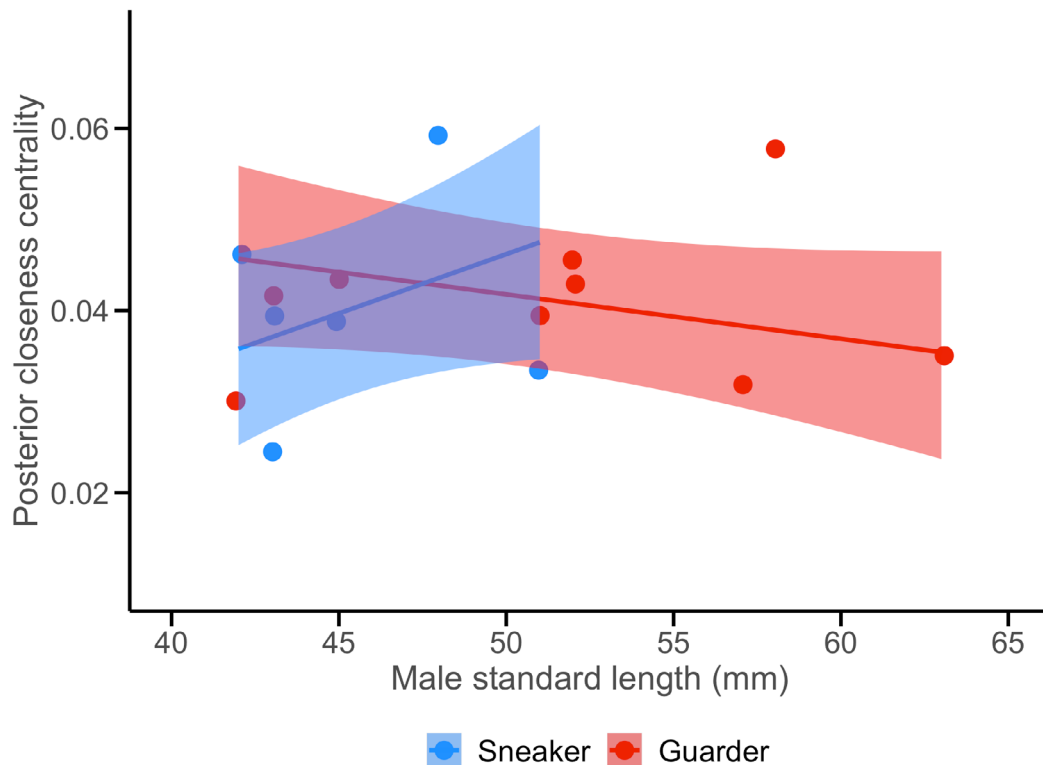


Fig. 4. Mean closeness centrality of male European bitterling as a function of male standard length (mm) and mating role, modelled using a Gaussian linear model for data collected in 2008. Shaded areas are 95% confidence intervals. Coloured points are observed data for different males.

sperm competition intensity; larger males would have more frequent opportunities to engage in sperm competition, particularly as “sneaky” matings in bitterling typically involve aggressive intrusions of territories, with larger males more capable of entering the territory of a rival and successfully releasing sperm over a mussel (Smith et al. 2002, 2004, 2009, Reichard et al. 2005, 2008). The model supported both predictions, with a statistically important interaction between mating role and body size (Table 3), with a body size negatively associated with sperm competition intensity in a guarder role and positively associated in a sneaker role (Fig. 5A). However, despite this result proving meaningful in considering the 95% credible intervals of the model, zero fell within the 99.5% credible intervals (not shown); a probability threshold that has been proposed as more appropriate for new findings to avoid false positives (Benjamin et al. 2018). Thus, our finding should be treated as “suggestive” rather than definitive.

Male mating polymorphisms are relatively well described in fish (Wootton & Smith 2015) and exemplified by anadromous salmonids. Male Atlantic salmon, *Salmo salar*, mature either as small “parr” or transform into smolts and migrate to sea, returning as large, mature adults. Mature parr rely on their small size and inconspicuousness to

achieve reproductive success. They possess relatively larger testes than migratory males and produce spermatozoa with greater swimming speed and ATP content (Vladić & Järvi 2001). Male *S. salar* that adopt different mating phenotypes also display divergent brain gene-expression profiles (Aubin-Horth et al. 2005). In bluegill sunfish (*Lepomis macrochirus*), males express a combination of fixed and sequential mating phenotypes. Some mature at approximately seven years, as large “parental” males construct nests, court females, and perform parental care. “Cuckolder” males, in contrast, mature at 2-3 years and “sneak” fertilisations. As they age, cuckolder males adopt a “satellite” tactic, whereby they express female colouration and behaviour and enter the nests of spawning parental males to attempt egg fertilisation as female mimics (Gross & Charnov 1980). In *R. amarus*, while mating tactics appear flexible, with males capable of switching among tactics, our results indicate that male mating role is determined, at least partially, by teste size and associated with specific mating behaviours.

The conditions that underpin male mating polymorphisms are relatively poorly understood. In *R. amarus*, environmental cues that elicit a switch between male mating phenotypes include density (Reichard et al. 2004a). At low male densities, the

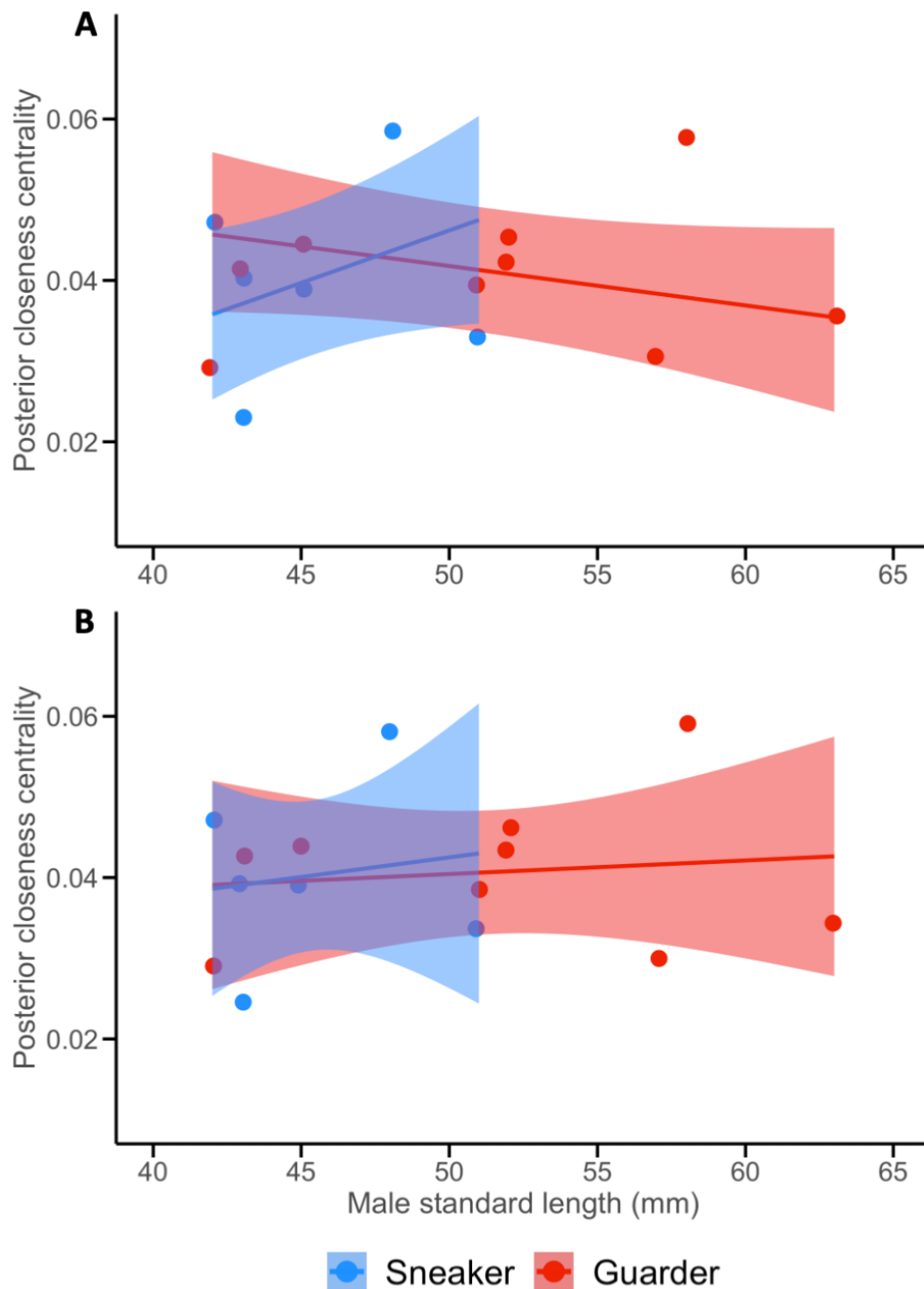


Fig. 5. Posterior mean closeness centrality of male European bitterling as a function of male standard length (mm) and mating role, modelled using a Gaussian linear model fitted using Bayesian inference with INLA; A) Model with informative priors; B) with uninformative (default) priors. Shaded areas are Bayesian 95% credible intervals. Coloured points are observed data for different males.

territorial tactic yields the highest reproductive success. As male density increases, the mean success of territorial males is eroded as more non-territorial rivals attempt to participate in matings. At a density at which the reproductive success of territorial behaviour is equivalent to non-territorial males, territoriality breaks down, and the mating

system switches to one of group spawning, with little aggression among males (Reichard et al. 2004b).

Because the growth pattern in fishes is indeterminate, growth rate and body size are key intrinsic factors that dictate life-history evolution (Wootton 1998), with direct implications for the male mating phenotype



(Wootton & Smith 2015). The role of growth rate in determining male mating phenotype in salmonids has long been recognised (Fleming 1996), and this conditional pattern of male mating status is probably widespread. Alternatively, males may express distinct growth patterns that dictate their mating role. This scenario is the case in the Mediterranean wrasse, *Symphodus ocellatus* (Alonzo et al. 2000), and perhaps in other species, though divergent growth patterns are rarely demonstrated. In the case of *R. amarus*, our findings imply a pivotal role of body size in male mating role, behaviour and position in a sexual network.

Pre-oviposition ejaculation is a striking feature of the European bitterling mating system. Given the apparent longevity of bitterling spermatozoa in the mussel mantle cavity, multiple ejaculations by males appear to function in sperm competition when multiple males participate in spawning. However, given the depletion of spermatozoa in the mussel mantle cavity (Smith & Reichard 2013) and the unpredictable timing of oviposition, a prediction is that guarder males should maintain a minimum density of spermatozoa in mussels in their territory, following Parker's (1998) concept of "topping up". Male bitterling do appear to systematically patrol mussels in their territory, as well as those of their rivals, examine the exhalant siphons of mussels and frequently ejaculate over them (Smith et al. 2004); a behaviour that is performed even in the absence of females; though the presence of a female with an extended ovipositor significantly increases the rate of inspection and ejaculation (Konečná et al. 2010). Mussel inspection by males may provide them with cues about the presence of spermatozoa in a mussel (Agbali 2011). The extensive network of overlapping ejaculates in spawning sites we observed justifies our adoption of network analysis as a tool for quantifying intrasexual competition in the bitterling mating system.

From the perspective of female mating decisions in European bitterling, if females attend to male ejaculation, they could potentially bias paternity towards a specific male. Female bitterling are sensitive to the presence of different numbers of males and perform behaviours that increase the probability that more than one male will participate in mating (Kanoh 2000, Smith & Reichard 2005). When offered the opportunity, female bitterling are choosy over the males with which they will mate. However, male dominance can override female choice, at least in an experimental setting (Reichard et al. 2005, Casalini et

al. 2009, Agbali et al. 2010). However, if females can match oviposition with preferred male ejaculations, they may be able to exert cryptic choice (Eberhard 1996) or at least to bias the probability of fertilisation of ova to particular males.

In the present study, we did not include females in the network since we focused solely on understanding male connections through overlapping ejaculates. However, if two or more males compete to fertilise a clutch of eggs deposited by a female in a mussel, those males are connected to the ovipositing female, and spawning females could be included in a sexual network of the sort we present here. However, given that female bitterling produces multiple clutches over a day, and multiple males may attempt to fertilise the eggs of the same female but not all her clutches, an alternative modelling approach is to connect competing males by egg clutches, rather than females or spawning locations. This approach of treating individual ovipositions as discrete nodes in the network will more directly capture male competition for fertilisations.

Bayesian inference is recognised as a tool for generating robust and reproducible findings (Miočević et al. 2020). A key feature of Bayesian inference is that it permits the incorporation of prior information in model formulation. Despite an acceptance of the value of Bayesian inference, models incorporating informative priors in ecology are rare (Banner et al. 2020); models instead tend to use uninformative or weakly informative priors and are often fitted with "default" priors. The failure to adopt informative priors may stem from an attempt at "objectivity" and a reluctance to fit models with "subjective" priors (Banner et al. 2020, Wesner & Pomeranz 2021). However, there is potentially substantial value to be gained from incorporating existing information as informative priors in models (e.g. Morris et al. 2013), particularly where data are sparse and prior information is directly exchangeable with new data. In the case of the present study, the exchangeability of the data used to generate priors was unambiguous since it was obtained at an earlier date with the same experimental facilities using animals collected from the same population. Data from the earlier study could not be directly pooled with later data for analysis since there was a risk of variance inflation due to collinearity between the year of data collection and the difference in the size distribution of males used on each occasion. However, utilising data from the earlier (2008) study to generate priors offered an opportunity to incorporate this information.



The model with informative priors yielded a result that diverged from a model with default priors. A sensitivity analysis illustrated that the model parameters were moderately sensitive to changes in prior information (Table S1, Fig. S7). However, given the provenance of the priors in this study, we can be confident that the findings reflect both the priors and data, which is the expectation of a Bayesian model that incorporates informative priors (Banner et al. 2020).

Bayesian inference offers an alternative framework to data analysis to the classical frequentist approach and has several advantages. One is that prior information can be incorporated into an analysis. Using prior information in a model is intuitively appealing and better reflects the scientific method of building on previous knowledge. A second advantage is avoiding hypothesis testing and *P*-values, which do not permit direct conclusions about model parameters to be drawn; only about hypothetical data that will never be collected.

The small number of males that were individually marked and followed over the subsequent four days of reproductive behaviour in our study might be perceived as a weakness, which would be the case in a frequentist setting since frequentist inference becomes more biased as the sample size decreases from infinity, and is markedly biased with small samples. However, Bayesian inference is unbiased with respect to sample size and can accommodate any sample size, no matter how small. However, one outcome of the small sample size was that GSI data were too coarse to decompose into more than two groups; guarder and sneaker. Based on behavioural data and previous measurements of GSI in natural populations (C. Smith, unpublished data), there is evidence for three discrete male mating roles in European bitterling, with an additional “pirate” role. Pirate males adopt a more cocksure approach to mating by actively guarding territories around mussels while simultaneously entering neighbours’ territories, temporarily usurping them from resident males, and releasing sperm over mussels. Males adopting this role tend to be large-bodied with a GSI intermediate between guarders and sneakers, typically 3.0-3.5 (see Fig. 2). In future studies, sampling a larger group of individually-marked males may permit males performing this role to be separated statistically from guarders and sneakers.

A potential weakness of the study arises from classifying males by mating roles based solely

on GSI. A conspicuous feature of the European bitterling mating system is that male mating roles appear flexible, at least behaviourally. Thus, while some males play some roles consistently, they can also switch among roles rapidly (Smith et al. 2004). Notably, male bitterling appear not to express marked individual correlations in behavioural traits (sometimes termed “personalities”) (Řežucha et al. 2012), which appears to be a feature of some animals, including fishes (e.g. Dingemanse et al. 2009).

European bitterling proved unusually amenable to research aimed at understanding mating system evolution using network analysis. Because bitterling must use freshwater mussels for reproduction, precise experimental control can be exercised over where and when spawning behaviour occurs. The quantity and quality of spawning sites can also be readily manipulated. Bitterling adapt freely to experimental mesocosms and aquariums and can be readily observed under natural conditions. Their mating system is well described; they perform unambiguous reproductive behaviours and express flexibility in mating behaviours. Bitterling also comprise a species-rich subfamily (Acheilognathinae) with well-resolved phylogenetic relationships (Chang et al. 2014, Kawamura et al. 2014), permitting robust comparative studies of mating system evolution among species. Alternative mating behaviour is also a fundamental feature of the mating system of bitterling; even in a situation where all males can potentially hold their own territory, sneak matings are common (Smith et al. 2004), making these fishes outstanding subjects for research on mating system evolution.

In conclusion, we used high-resolution behavioural data to quantify sperm competition intensity among competing male European bitterling in a large mesocosm. In addition, we modelled sperm competition intensity among males adopting alternative mating roles to understand how sperm competition intensity varied between roles as a function of body size. We demonstrated that larger males performing a guarder role experienced lower sperm competition intensity than smaller males, while the reverse was true for sneaker males, with larger males experiencing greater sperm competition intensity. These findings matched our predictions, which we discuss in the context of the bitterling mating system. Finally, we demonstrate that the bitterling-mussel system offers an unusually tractable model system for understanding mating system evolution through a sexual network approach and the value of

Bayesian inference for modelling small datasets with informative priors.

Acknowledgements

Experimental work was approved by the Ethical Committee of the Institute of Vertebrate Biology (no. 79/2017) and by the Ministry of Agriculture (CZ 62760203) following the legal regulations of the Czech Republic and European Union. The study was supported by a Czech Science Foundation grant 21-00788X. We are grateful to David Fairhurst for assistance with data collection.

Author Contributions

M. Reichard, C. Smith and R. Spence designed the study and collected the data. R. Bailey and C. Smith analysed the data. Finally, C. Smith drafted the manuscript; all authors read and contributed to the final version.

Data Availability Statement

The data and RScript supporting this study's findings are available in the FigShare Digital Repository: <https://doi.org/10.6084/m9.figshare.21707849>.



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Supplementary online material

Fig. S1. Posterior and prior distributions for fixed parameters of Bayesian linear regression to predict the closeness centrality of male European bitterling in a sperm network. The model is fitted with default (non-informative) priors. Distributions for: A) model intercept (β_1); B) slope for male standard length (β_2); C) slope for mating role (β_3); D) slope for the interaction of male standard length and mating tactic (β_4). The solid black line is the posterior distribution, the solid grey line is the prior distribution, the grey shaded area encompasses the 95% credible intervals, the vertical dashed line is the posterior mean of the parameter, and the vertical dotted line indicates zero. The parameter is considered statistically important for parameters where zero (indicated by a dotted line) falls outside the 95% credible intervals (grey-shaded area) range.

Fig. S2. Posterior and prior distributions for the standard deviation of the hyperparameter of Bayesian linear regression to predict the closeness centrality of male European bitterling in sperm network. The model is fitted with default (non-informative) priors. The solid black line is the posterior distribution, the solid grey line is the prior distribution, the grey shaded area encompasses the 95% credible intervals, and the vertical dashed line is the posterior mean.

Fig. S3. Posterior and prior distributions for fixed parameters of Bayesian linear regression to predict the closeness centrality of male European bitterling in a sperm network. The model is fitted with informative priors. Distributions for: A) model intercept (β_1); B) slope for male standard length (β_2); C) slope for mating role (β_3); D) slope for the interaction of male standard length and mating tactic (β_4). The solid black line is the posterior distribution, the solid grey line is the prior distribution, the grey shaded area encompasses the 95% credible intervals, the vertical dashed line is the posterior mean of the parameter, and the vertical dotted line indicates zero. The parameter is considered statistically important for parameters where zero (indicated by a dotted line) falls outside the 95% credible intervals (grey-shaded area) range.

Fig. S4. Posterior and prior distributions for the standard deviation of the hyperparameter of Bayesian linear regression to predict the closeness centrality of male European bitterling in sperm network. The model is fitted with informative priors on the fixed effects and a default prior on the hyperparameter. The solid black line is the posterior distribution, the solid grey line is the prior distribution, the grey shaded area encompasses the 95% credible intervals, and the vertical dashed line is the posterior mean.

Fig. S5. A) Frequency histogram; B) Uniform Q-Q plot with confidence bands (shaded) for cross-validated probability integral transform (PIT) values of the Bayesian linear model.

Fig. S6. Bayesian residuals plotted against: A) model fitted values; B) male standard length (mm), and C) mating role, to assess the homogeneity of residual variance of the Bayesian linear model.

Fig. S7. Posterior distributions for parameters of a Bayesian linear regression to predict the closeness centrality of male European bitterling in a sperm network. Distributions for: A) model intercept (β_1); B) slope for male standard length (β_2); C) slope for mating role (β_3); D) slope for the interaction of standard length with mating role (β_4). The solid black line is the posterior distribution for the optimal model, the dashed grey line is the posterior distribution for an alternative model with the priors increased by 20%, and the dotted grey line is the posterior distribution for an alternative model with the priors decreased by 20%.

Table S1. Sensitivity analysis for a 20% increase and decrease in priors on fixed effects and the % change in the posterior mean. CrI is the Bayesian 95% credible interval.

Table S2. Posterior mean estimates for parameters of a Bayesian linear regression to predict the closeness centrality of male European bitterling as a function of male standard length (mm) and mating role modelled using a Gaussian LM fitted using Bayesian inference with INLA for 2008 data. CrI is the Bayesian 95% credible interval. The parameter σ is the standard deviation of the residuals.

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