Aquatic Botany 140 (2017) 34–37

Contents lists available at ScienceDirect

Aquatic Botany

journal homepage: www.elsevier.com/locate/aquabot

Review

# An overview on the effects of fish consumption on seed germination: Pitfalls, challenges, and directions

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ARTICLE INFO

*Article history:*
Received 7 April 2016
Received in revised form 5 January 2017
Accepted 17 January 2017
Available online 20 January 2017

*Keywords:*
Experimental design
Ichthyochory
Seed dispersal
Floodplain
Seed fates
Germination experiments
Feeding trials
Seed fate

Although traditionally neglected in studies of animal-plant interactions, fish are nowadays considered important seed dispersers of aquatic, riparian and floodplain plant species (Correa et al., 2015b). Interestingly, since the pioneering studies on seed dispersal by fish (i.e., ichthyochory), authors have not only described fruit consumption by fish in flooded forests, but have also investigated how frugivorous fish may affect plant fitness by dispersing seeds. Specifically, classic and contemporary studies used experiments to explore how the passage of seeds through a fish's digestive tract can affect germination success and/or speed (see Correa et al., 2007; Pollux, 2011).

Experimental studies have shown that tropical and temperate fishes can keep seeds intact and viable after ingestion, break dormancy, enhance germination success and alter germination speed (see Correa et al., 2007, 2015b; Pollux, 2011). Recent experiments have also assessed how fish and/or seeds traits can influence germination outcomes and, ultimately, seed dispersal efficiency (Anderson et al., 2011; Boedeltje et al., 2015; Correa et al., 2015a; Pollux, 2016). Although the number and complexity of experimental studies about seed dispersal by fish have been increasing over the last two decades, there is a large variation in the experimental design and protocols between studies, a scenario that makes difficult a synthesis and discussion of general patterns in the field (Pollux, 2011). Several authors have recently pointed out a demand for a more standardized protocol for studying seed dispersal by fish (Horn et al., 2011; Pollux, 2011; Correa and Anderson, 2016).

Here, I present an overview of the design and protocols of experiments concerning how fish consumption can affect seed germination. I discuss current pitfalls on experimental designs and suggest some directions that can improve the scientific gains and the methodological standardization in future studies. Overall, I call attention for an experimental ecology in the field of seed dispersal by fish that is biologically meaningful and grounded in the natural history of the target fish-fruit interaction.

The first point to be considered, even before setting up germination experiments, is whether the studied fish species is indeed a potential seed disperser. Biologically, it is more meaningful to set up germination tests when the frugivorous species i) consumes a substantial quantity of fruits/seeds, ii) retains a representative proportion of seeds fully intact after digestion and/or iii) deposits the seeds in suitable sites for germination. Recently, some studies proposed to select plant species to feeding trials based on morphological traits (e.g. fruit/seed size) and their presence in habitats

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http://dx.doi.org/10.1016/j.aquabot.2017.01.005

where fish can act as seed dispersers (e.g. Boedeltje et al., 2015). However, beyond this indirect evidence (that can only indicate species potentially consumed by fish, not necessarily the consumed or dispersed ones), field observations and dietary studies (e.g. stomach content analysis or stomach flushing during fructification periods) are essential to confirm if the consumption of fruits by fish is actually representative in the studied system. Prior natural history knowledge about the fish-fruit interaction may also reveal aspects of seed dispersal that can be considered in the design of experiments, such as the proportion of masticated seeds and the foraging behavior of the fruit-eating fish (Costa-Pereira et al., 2011). In this sense, it is important first to have strong naturalistic evidence that the fish species consumes the fruits (not only occasionally) and keeps the seeds intact (e.g. dietary studies, naturalistic observations), and then design the experiments and interpret their outcomes in light of other qualitative and quantitative aspects of seed dispersal (e.g. proportion of intact seeds, suitability of deposition sites).

Another relevant point is how fruits are offered to fish and how seeds are recovered for germination experiments. In some studies aiming to retrieve seeds after their passage through the entire digestive tract, seeds were offered embedded in dough pellets to the fish during feeding trials (e.g. Pollux et al., 2007; Boedeltje et al., 2016). Although this may be the only way to feed some fish in captivity situations, offering natural fruits or seeds is certainly a more realistic protocol. This is relevant because the presence of an artificial material embedding the seeds (e.g. dough) may affect how seeds are processed along the digestive tract, particularly in oral cavities, where there are complex food selection mechanisms (Pollux, 2011). In turn, in another set of studies, wild-individuals were killed and seeds recovered from their stomach contents for germination experiments. Traditionally, seeds retrieved from stomachs are considered inappropriate for seed germination tests, as they are only a subset of the complex mechanical and/or chemical actions happening during the passage through the entire digestive tract (Schupp et al., 2010). However, recently Correa and Anderson (2016) showed that seeds collected from the stomach via lavage and seeds that passed through the entire digestive tract might present similar germination patterns. In this sense, seeds from stomachs should be used carefully in germination experiments, preferably after a pilot study comparing their germination efficiency with defecated seeds (Correa and Anderson, 2016). Seeds collected from stomachs are usually also appropriate to taxonomically identify the consumed fruit species (mainly when they are intact enough, e.g. still have pulp) and to quantify the rate of seed mastication.

Interestingly, Correa and Anderson (2016) call attention to the use of non-lethal sampling of stomach contents and quantification of seed dispersal by frugivorous fish. These authors proposed a standard protocol for a lavage method, which successfully recovered more than 90% of stomach contents in the pacu (*Piaractus mesopotamicus*). This is a timely discussion as some important frugivorous fish have suffered drastic reductions in population size in recent decades, such as the tambaqui (*Colossoma macropomum*) (Costa-Costa-Pereira and Galetti, 2015). In this sense, future studies in the field should privilege the use of stomach flushing and other non-lethal techniques, such as setting up enclosures in natural habitats (e.g. cages in floodplains) or keeping fish individually in suitable plastic bags, checking periodically for defecated seeds (Silveira and Weiss, 2014). Some of these non-lethal approaches also allow quantifying the gut retention times, which can be applied to model seed dispersal distance (Anderson et al., 2011; Van Leeuwen et al., 2016).

Interpretability of the results is also essential. Many different indexes and statistical approaches have been proposed to quantify the germination process (Ribeiro-Oliveira and Ranal, 2015), but authors should opt for the use of intuitive and biologically meaningful ones. For instance, many studies, not only in the field of seed dispersal by fish, use the Maguire's index (1962) to quantify differences in germination speed of control *vs*. consumed seeds. This measure (also referred as Germination Speed Index, GSI) is widely used by agronomists and foresters in germination experiments to quantify the vigor of seedlings (Ribeiro-Oliveira and Ranal, 2015). However, this index has received a lot of criticism, particularly in ecological studies, mainly because it has no clear biological meaning (Brown and Mayer, 1986, 1988). For instance, Yule et al. (2016) observed that control seeds of *Banara arguta* (Salicaceae) have a germination speed index twice the rate of seeds consumed by the freshwater sardine (*Triportheus nematurus*) in the Pantanal wetlands. Does this statistically significant difference in GSI values have important biological consequences? Does this difference reflect a delay of germination on a meaningful timescale? If the lower GSI value represents a few days of delay in the germination of a small proportion of seeds, the freshwater sardine will not positively affect the recruitment of *B. arguta*, as the flood season lasts for several months in the Pantanal wetlands. Thus, quantifying the germination process in biologically meaningful measures is essential to contextualize if the observed effects on germination rate can be truly relevant in field conditions. The rate of germination, cumulative germination, survivorship analysis (Anderson et al., 2009) or the novel quantitative approach taken by Boedeltje et al. (2016) are good examples.

Generally, an important virtue of a germination experiment is realism. In this sense, control and treatments should be designed considering the environmental conditions in which seeds are subjected in the natural habitats. Commonly, the effect of the consumption by fish on seed germination is tested via a comparison between the germination of seeds without pulp (pulp manually removed, control) *vs*. a treatment of seeds that have passed through the gut (or recovered from the stomach). However, there are other possible fates of seeds in natural habitats that are not considered in this design. For instance, it is common in nature for seeds to remain inside fruits, which can be dispersed by water. Thus, 'germination from intact fruits' (Fig. 1, ii) should be an important treatment to be included in germination trials (Samuels and Levey, 2005; Pollux, 2011). In this case, a comparison between intact fruit (control 1) *vs*. consumed seeds allows quantifying the overall effect of the fish consumption on the germination process. A comparison between intact fruit (control 1) *vs*. manually extracted seeds (control 2) allows evaluating the simple effect of the pulp removal on germination. Finally, a comparison between manually extracted (control 2) *vs*. consumed seeds quantifies the effects caused by the complex biomechanical and chemical processes acting along the passage through the gut, regardless the effect of pulp removal.

Another important aspect neglected in experimental studies of seed dispersal by fish is to include submerged treatments (Fig. 1). For example, submersion is necessary to break dormancy of seeds of Amazonian species that remain submerged for several months after dispersal (Kubitzki and Ziburski, 1994). The submergence of seeds during a period biologically coherent (e.g. the duration of the flood season after the dispersal period) represents an alternative to subject propagules to conditions similar (e.g. temperature, water column height) to those imposed by seasonally flooded habitats. Although this is an approach that has been used in studies aiming to test the tolerance of seeds to flooding (Lucas et al., 2012; de Melo et al., 2015), it remains rarely used in studies of seed dispersal by fish (e.g. Kubitzki and Ziburski, 1994).

Measures of germination success and rate quantified under controlled laboratory conditions are likely different from those in natural habitats. In the field conditions are usually harsher, and the biological interactions that can affect germination process are complex and highly variable (e.g. competition, parasitism, facil-

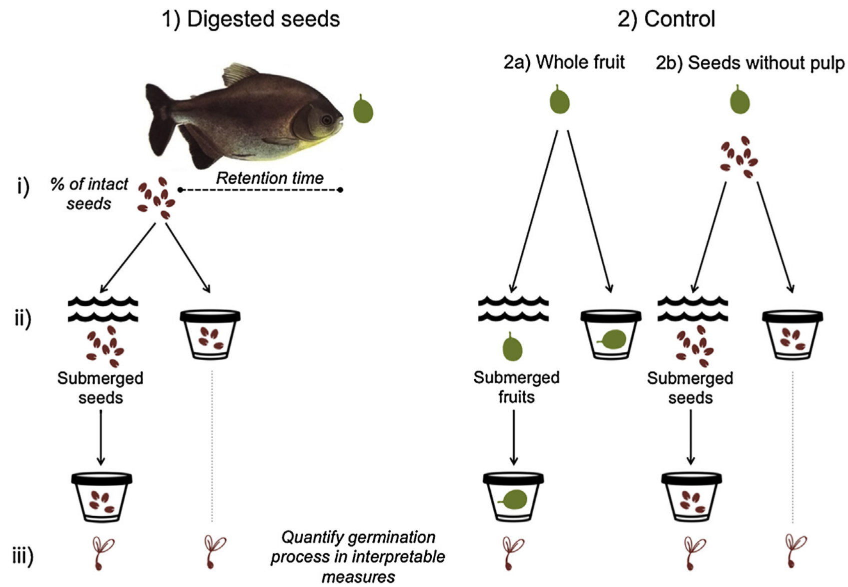


**Fig. 1.** Do fish affect germination processes? A framework to investigate how fish consumption might influence seed germination. First, (i) as germination is only one of many aspects that involve seed dispersal, other aspects should be considered and measured during experiments (e.g. proportion of masticated seeds, retention time). Seeds consumed by fish can be masticated or be deposited intact on floodplains. (ii) Submergence treatments (during realistic time periods, e.g. the time difference between early fruiting and flood duration) are recommended both for digested and control seeds. (2a) Include a whole fruit treatment is also recommendable because seeds that are not consumed by fish (control) can remain inside whole fruits. (iii) Finally, the germination process should be quantified in biologically meaningful measures, i.e. taking into account as much the natural history of the fish and plant species investigated and the environmental conditions encountered in the studied habitat.

itation, predation) (Verdú and Traveset, 2005; Pollux, 2011). In this context, an option is to set up germination experiments in a field (e.g. experiments set in floodplains) or a greenhouse using local conditions (e.g. soil, temperature, moisture) instead of artificial laboratory conditions (e.g. Anderson et al., 2009; Correa et al., 2015a). Correa and Anderson (2016) pointed out that setting up a germination experiment in the field can be logistically simple and cheap.

An advantage of using field experiments is the improvement of the applicability of the results to support conservation initiatives, such as the reintroduction of frugivorous species in overfished sites, like some Amazonian flooded forests that have been heavily exploited in recent decades (Costa-Costa-Pereira and Galetti, 2015). On the other hand, the accuracy of laboratory experiments (or experiments in the field under controlled conditions) can reveal precise measures about germination process. In this sense, integrating the virtues of field and laboratory experiments is an important challenge for future studies in the field.

The main messages of this opinion paper can be summarized as follows: i) experiments on fish-fruit interactions should be grounded in natural history; ii) offering fruits and retrieving seeds should be as natural as possible and, if feasible, following non-lethal protocols; iii) experimental design should consider the realistic potential fates of seeds (e.g. submersion and germination from intact fruits) and coherent time scales (e.g. duration of the flood season); iv) the germination process should be quantified by biologically meaningful measures; v) germination results should be interpreted in light of the characteristics of the studied system, considering other quantitative and qualitative components of seed dispersal (e.g. proportion of masticated seeds, suitability of deposition sites). Indeed, setting up precise, general and realistic experiments is a long-standing challenge in ecology (Hairston, 1989) and in experiments of seed dispersal by fish, this is not different. Ecologists in this field should keep in mind that some simple precautions in the experimental design and used protocols might provide more standard, applicable and biologically meaningful conclusions.

## Acknowledgments

I am grateful to the three Aquatic Botany editors for their constructive and thoughtful suggestions<, to Larissa SM Sugai for the insightful comments on the manuscript, to Ben Dickson for the English review, and to São Paulo Research Foundation (FAPESP) for grant #2014/20924-5.

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