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Research Article

Evaluation of the Photocopy Method for Counting *Puntius conchonius*'s Eggs

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Abstract: The photocopy technique was developed to count fish eggs. Fecundity of the rosy barb *Puntius conchonius* was determined by both actual counts and the photocopy method. Egg stripping and fertilisation were performed manually. After fertilisation, water-hardened eggs were photocopied. White circles were counted and recorded as viable eggs and dark dots counted as dead eggs. Egg numbers estimated by this technique were significantly correlated with those estimated by actual counting ($r = 0.999$; $P < 0.05$). The advantage of the photocopy method is that it is a relatively fast method without significant loss in accuracy and it is cheaper than the other currently used methods.

Key Words: Egg number, photocopy technique, rosy barb, *Puntius conchonius*

Puntius conchonius Yumurtalarının Sayımında Fotokopi Yönteminin Geliştirilmesi

Özet: Balık yumurtalarını saymak için fotokopi tekniği geliştirilmiştir. Konkinyus *Puntius conchonius*'un yumurta verimliliği, gerçek sayım ve fotokopi yöntemiyle belirlenmiştir. Yumurta sağımı ve dölleme el ile yapılmıştır. Döllemeden sonra, su ile sertleşen yumurtaların fotokopileri alınmıştır. Beyaz daireler sayılıp canlı yumurtalar, siyah noktalar ise sayılıp ölü yumurtalar olarak kaydedilmiştir. Bu teknikle hesaplanan yumurta sayıları ile gerçek sayım arasında önemli ölçüde korelasyon saptanmıştır ($r = 0,999$; $P < 0,05$). Fotokopi yönteminin avantajı, doğrulukta önemli ölçüde bir kayıp olmaksızın, hızlı ve aynı zamanda kullanılan diğer metotlardan ucuz olmasıdır.

Anahtar Sözcükler: Yumurta sayısı, fotokopi tekniği, Konkinyus, *Puntius conchonius*

Introduction

Puntius conchonius is a colourful tropical freshwater aquarium fish that lives in rivers, streams and other freshwater environments, and is used for ornamental and experimental purposes (1). It is a prime candidate for Cypriniform of the family Cyprinidae. The generic name of this species, Barbus or Puntius, is still under discussion (1). They can be propagated inexpensively and under optimum conditions they produce large numbers of eggs (2,3).

It has been used extensively as a model fish in fish physiology (4), fish disease (5), genetics (6), fish behaviour (7), cryobiology (2) and so on. Varadi et al. (6) pointed out that model fish should have a short generation time and thus can be used easily for genetic manipulations and *P. conchonius* is therefore well suited for experimental studies as a model fish. Thus, in this study, it is also used as a model fish for an application of a new method for fecundity measurement.

Many different methods have been used to estimate fecundity (8-12). The two most common methods still in use to count fish eggs involve digesting the ovaries in a mercury-based fixative (usually Gilson's fluid) and counting a subsample of the eggs on the basis of the certain volume (the volumetric method (8)) or the certain weight (the gravimetric method (9)).

Several applications of these methods have been described. The fecundity, for example, has been studied by a modification of the area method (13). The gonads were disintegrated in Gilson's fluid, and poured into a

petri dish of a specified area. The eggs were then settled by gravity subsample obtained with a tube of known area, and the eggs were counted and measured under a stereomicroscope by an ocular micrometer (1 mm = 20 micrometer units). After measurements, the subsample was returned to the whole sample and a further subsample was taken. Some other adaptations of volumetric and gravimetric methods have been described by Musonda (14), Gundersen et al. (15) and Hinshaw and Thompson (16).

In addition to these methods mentioned above, several automated methods for counting eggs have also been described (17,18), but these methods count only over a limited egg size classes.

Recently, quantitative (stereological) methods for analysing histological sections of gonads have been introduced (19-21). These methods are based on the assumption that as only mature oocytes are shed from the fish at each spawning time, the number of mature oocytes in the ovary can be estimated as fecundity.

A simple photographic technique has been used by Murdoch et al. (10). Use of a camera in the laboratory or in the field to count fish eggs is expensive in all instances because of disposable films and photographs.

Post-ovulatory follicles have also been used to indicate amount of laid eggs (12). In this technique some measurement errors are inevitable because often their regression occurs very quickly (22).

All these methods have their limitations: Gilson's fluid is highly toxic, the digestion process takes several months and the samples need to be shaken frequently. The method described by Witthames and Walker (17) can only be applied to eggs with a diameter greater then 200 µm

The stereological method requires qualified staff and expensive equipment and a specialist knowledge of cell structure, and so it is impractical for fish farmers.

The information available on *P. conchonius* is based on the observations of aquarists or hobbyists. It is only recently that laboratory studies have begun to broaden our knowledge of *P. conchonius* in terms of fecundity. The method described here has not been applied to *P. conchonius* or any other fish species; therefore the aim of this trial was to describe the application of a simple photocopy method for counting fish eggs. The method has been applied to *P. conchonius* eggs and compared with actual counts of egg numbers.

Materials and Methods

Experimental Design

Facilities of the Institute of Aquaculture, University of Stirling, Stirling, Scotland, were used to conduct the experiment. A closed recirculating system consisting of 4 circular Fiberglas tanks of 20-l capacity (20 x 40 x 35 cm) was used. The water temperature of each tank (27 ± 1 °C) was maintained by a 50-watt electric heater. *P. conchonius* of known age (6 months old), originating from one pair of spawners, were stocked into each tank at the rates of 7 females and 4 males. The mean length and weight of 28 females were measured as 5.39 ± 0.41 cm and 4.12 ± 0.93 g, respectively. The duration of the experiment was 6 months.

During the experimental work, fish were fed with 2 different feeds: trout pellets (Trouw, UK Ltd.) and flakes (Ewos Ltd., Bathgate, Scotland) 3 times a day. Because in the recirculatory water system biological filtration was available, water quality parameters were taken weekly.

Application of the Method

In order to perform artificial fertilisation, courtship behaviour was checked daily in the early morning by observing the continuous chasing of the female by the male. Paired males and females had been selected and placed in a 200 ml beaker and were anaesthetised individually by using 2-phenoxyethanol at a concentration of 1:20,000 in water (Sigma Chem. Co., Dorset, UK). Stripping of eggs was accomplished manually by pressing the abdomen in the direction of the caudal fin. Then the eggs were collected in medium size petri dishes (9 cm in diameter). Milt collection was carried out directly by using 25 µl capillary tubes. After stripping, the females and males were immediately placed in a recovery tank containing fresh aerated water at an oxygen concentration of 7-8 mg/l. Fertilisation was performed by mixing the eggs and the sperm, adding water and shaking the petri dish by hand. Excess sperm was washed off with the same temperature water from the brood stock tanks and the eggs left for 10 minutes to get water-hardened. After being fertilised and water-hardened, the eggs were photocopied and a piece of thick white paper was used to cover the petri dish while the eggs were photocopied. White circles were counted and recorded as viable eggs, and dark dots counted as dead eggs.

During the photocopy process eggs were suspended in the water and for each batch they were counted by both

the photocopy and manual method to assure accuracy of this method.

Paired sample t-test and regression analysis were used (23) to compare the two techniques. The results were plotted using the Sigma plot graph program.

Results

During the experimental studies water quality parameter were measured (Table).

The number of eggs produced in a single spawning time (batch fecundity) differed widely between individuals. The number of eggs ranged from 198 to 1698 in fish between 4.65 and 5.99cm in length and 2.26 and 5.42 g in weight. They spawned throughout the year with large numbers of viable, semi-adhesive, not adhesive, and transparent eggs. The development period from fertilisation to hatching was found to be 24 h at 28 °C. The main diameter of their eggs ranged from 0.974 ± 0.037 to 1.123 ± 0.028 mm.

The photocopy and actual counting methods were compared using fertilized eggs from the same pair of fish. The results showed that the photocopy method is as accurate as the manual counting method (Figure). Hence, attempts to determine the fecundity of *P. conchonius* using the photocopy method showed that this method was successful at $P < 0.05$ level $r = 0.999$. The regression models of actual counts versus photocopy method counts were highly significant ($P < 0.05$ level; $r = 0.999$), (Figure).

When paired sampled t-test was used, it was shown that the photocopy counting method resulted in less count with the average of 20 eggs ($P > 0.01$).

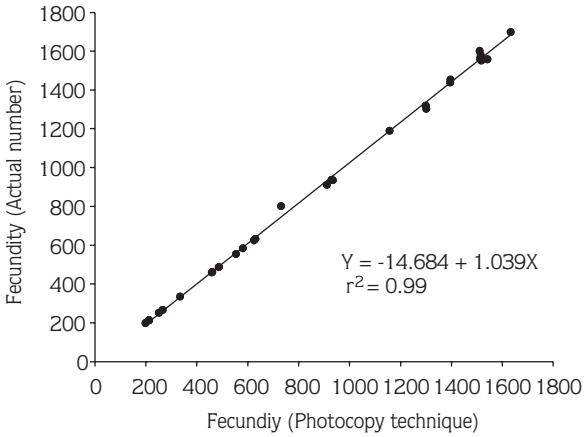

Figure. Comparisons of two techniques for estimation of fecundity of *P. conchonius*, raw data. Each point indicates a single female, n = 28.

Discussion

Fecundity and egg quality of *P. conchonius* were studied by Axelrod (24), although this study was based on his observations and was performed in the natural environment. The only reported study of the egg traits of *P. conchonius* was that by Varadi and Horvath (25). In their study, *P. conchonius* eggs were found to be transparent and semi-adhesive. Similar results were recorded in the present study when compared to these studies. Varadi and Horvath counted the number of eggs, after in vitro fertilisation in swollen, water hardened state (actual counting method). They pointed out that the number of eggs produced in a single spawning time differed widely between individuals (450-1500 per female). In our study, the number of eggs ranged from 198 to 1696. This result is similar to that of Varadi and Horvath (24).

Table. Water quality parameters during the experimental studies, mean ± sd.

Time (months)	T (°C)	Oxygen (mg/l)	pH (mg/l)	NO_2^- (mg/l)	NO_3^- (mg/l)	NH_4^+ (mg/l)
April	27 ± 0.7	7.6 ± 0.2	7.4 ± 5.021E-	0.0	20	025
May	27 ± 1.1	7.6 ± 0.2	7.4 ± 5.084E-	0.0	20	0.25
June	27 ± 0.8	7.6 ± 0.3	7.4 ± 5.886E-	0.0	20	0.25
July	27 ± 1.1	7.7 ± 0.2	7.4 ± 7.134E-	0.0	20	0.25
August	27 ± 0.7	7.6 ± 0.3	7.4 ± 7.215E-	0.0	20	0.25
September	27 ± 0.9	7.7 ± 0.3	7.4 ± 4.311E-	0.0	20	0.25

Witthames and Walker (17) described a method, an automated particle counter, for counting all of the eggs in the ovaries of plaice, *Pleuronredes platessa*. Joyce and Ramson (18) counted eyed eggs by an electronic fish counter. However, the automated particle counter and electronic fish counter count dead eggs and cannot count all egg size classes, thus giving misleading fecundity measurements. In the photocopy method dead eggs are certainly not counted but all egg size classes are counted.

Due to the stickiness of the eggs, the photocopy counting resulted in an average of 20 fewer eggs compared to the actual counting. Significant errors are only likely to occur in the photocopy method if the eggs have not been properly separated. Therefore two or more eggs are counted as one. However, the photocopy method can be used with confidence since preventing the stickiness of the eggs eliminates this difference. All eggs were counted by separating them from each other.

Some mortality was gradually seen in the photocopy technique. The reason for these mortalities might be as follows: (a) the water temperature might not be constant during photocopying, (b) the colour and density of light might be harmful to the eggs, (c) the nature of their semi-stickiness and (d) amount of water in the petri dish might be was not enough and (e) the size of petri dish is thought to affect mortalities since when the size was too small the eggs were not suspended very well in the water.

It was clearly observed that when the amount of water in the petri dish was sufficient, which means that eggs were suspended in the water, mortalities were very low. Hence, the present study suggests that the photocopy technique can be used to count fish eggs.

The photocopy counting method has the following distinct advantages over the volumetric, gravimetric, stereological and automated particle counter methods: (a) almost all automatic counters require Gilson's fluid, which is highly toxic, and so using the photocopy method the toxicity and long digestion period associated with Gilson's fluid could be avoided, (b) most of the automatic counters are expensive ($12,000-14,000) (4), whereas the photocopy machine used in this study is quite cheap ($640), (c) the photocopy machine make it possible to avoid counting dead eggs, which turn white as a result of yolk coagulation and are seen as blank dots on the paper. Therefore only viable eggs are counted, (d) and finally, special knowledge, and a specialist are needed to use the automatic counters and stereological techniques, but this is not convenient for the fish farmer, whereas the photocopy method does not need special knowledge or a specialist and is convenient.

Fish farmers and managers can plan properly their production capacity if they are able to count the eggs accurately and precisely. The photocopy machine provided reasonable counts of small *P. conchonius* eggs and could be equally applicable to other commercially important fish species that produce large numbers of small eggs. The method is cheaper, quite accurate, and faster than the methods commonly used currently.

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