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An optimised experimental test procedure for measuring chemical effects on reproduction in the fathead minnow, *Pimephales promelas*

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Abstract

The production of viable offspring is fundamental to the survival of any population. Tests that quantify effects on reproduction can, therefore, inform on the potential for long-term health effects of exposure to endocrine active chemicals. Surprisingly little is known, however, about the reproductive capacity of laboratory fish species used for chemical testing. As an example, the fathead minnow, *Pimephales promelas*, is widely used in chronic assessments of reproductive toxicology, and is readily induced to reproduce in captivity, yet there is little agreement on the reproductive capacity (egg number) of this species. For this species, the notable variation in reported estimates of egg number might relate to differences in the methods of egg collection adopted by many laboratories. To investigate this hypothesis, reproduction was assessed in a total of 200 pair-breeding fathead minnow, using egg collection methods that included the addition of trays placed beneath an inverted U-shaped PVC tile that is conventionally used alone for egg collection. The results demonstrated that the placement of a mesh-screened egg collection tray, beneath the spawning tile, increased estimates of the egg number by 25–67%. In addition, adopting the mesh-screened tray reduced variation in egg number between pairs, within an experiment, from >50% to <30% and variation between experiments was reduced from 53% to 7%. Adoption of the revised system for egg collection shows that egg number in the fathead minnow is considerably more consistent than frequently reported and is a highly robust endpoint against which chemical effects can be challenged effectively.

Keywords: Egg number; Reproductive toxicology; Fathead minnow; Egg collection

1. Introduction

A number of chemicals that are discharged into the aquatic environment have the capacity to interact with the endocrine system of fish and alter sexual development (Van Aerle et al., 2002; Kiparissis et al., 2003; Papoulias et al., 2003; Angus et al., 2005) and reproductive function (Harries et al., 2000; Ankley et al., 2001, 2002, 2003; Jensen et al., 2004). To understand the consequence of exposure to these chemicals, tests are required that can inform on their long-term health effects for aquatic organisms. The most comprehensive test available is the fish full life cycle test that is currently used in support of regulatory programmes in both Europe and North America (US EPA, 1986). Full life cycle tests, however, are costly, time-consuming and difficult to maintain. The focus is, therefore, moving to the development of short-term tests that encompass life stages vulnerable to the effects of EACs, such as the period of sexual differentiation during early life (Van Aerle et al., 2002; Andersen et al., 2003) or reproduction in adults (Kime and Nash, 1999; Harries et al., 2000; Ankley et al., 2001; Van den Belt et al., 2001). Studies on reproduction are particularly desirable as they have clear population relevance; a reduced reproductive capacity ultimately results in a reduction in the number of viable offspring. The reproductive capacity (egg number) of fish, however, is thought to be highly variable between individuals within a species (Bagenal, 1967). This can complicate assessments of egg number making it difficult to define control limits for which departures from expectation can be determined.

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In adult fish reproduction assays, the inherent variability in estimates of egg number, between individuals, can be addressed through the use of repetitively spawning fish species (e.g. the fathead minnow, *Pimephales promelas* (Harries et al., 2000; Ankley et al., 2001) and the zebrafish, *Danio rerio* (Kime and Nash, 1999; Van den Belt et al., 2001)). Through the use of such species, baseline data for the number of eggs spawned over a pre-exposure period can be determined, and compared with the number of eggs spawned over an equivalent chemical exposure period. Thus, altered reproductive capacity can be determined at the level of the individual and the variability in reproduction between individuals accounted for. Such a test design, however, is based on the assumption that egg number can be accurately quantified and that it would be constant over the duration of the study. This has yet to be adequately demonstrated for any laboratory maintained fish species.

One of the most widely used test species in chronic life cycle and early life-stage tests (US EPA, 1982, 1986, 1987, 1994; OECD, 1992) is the fathead minnow (Cyprinidae). More recently, the fathead minnow has also been used extensively to assess the effects of chemicals on reproductive function (Harries et al., 2000; Ankley et al., 2001; Länge et al., 2001; Jensen et al., 2004; Bringolf et al., 2004; Parrott and Blunt, 2005). In most laboratories, egg production rates for the fathead minnow have been reported to be between 2.5 and 26 eggs/female day (Ankley et al., 2001; Länge et al., 2001; Jensen et al., 2001, 2004; Bringolf et al., 2004; Pawlowski et al., 2004; Parrott and Blunt, 2005). In two laboratories, however, egg production rates have been reported to be considerably higher (between 73 and 109 eggs/female day (Gale and Buynak, 1982); between 43 and 112 eggs/female day (Harries et al., 2000)). Although many experimental factors could contribute to the different egg production rates, one notable difference is that in the two laboratories that report a higher egg production, a screened egg collection tray was placed under the inverted U-shaped tile to collect eggs not adhering to the tile (Gale and Buynak, 1982; Harries et al., 2000). All of the studies that reported lower egg production rates similarly used an inverted U-shaped tile to collect the spawned eggs, but did not include the use of a screened egg collection tray to collect eggs not adhering to the tile.

This study set out to establish an optimised method for egg collection in laboratory maintained fathead minnow, to provide greater accuracy in chemical testing using egg production as an endpoint. The first experiment (experiment I) determined variations in estimates of egg number derived using different egg collection strategies. Estimates of egg number determined using a PVC spawning tile only, were compared with estimates derived using the spawning tile together with an egg collection tray system (that was either screened – allowing eggs to fall into the tray, but preventing predation of those eggs by the adult fish – or unscreened). In the next series of experiments (experiments II–IV), using the optimised egg collection method, comparisons were made between three different stocks of fish to assess the level of variation in egg production both within an experiment (fish of the same age and similar weight) and between experiments (fish of different ages and weights), under the experimental conditions described. The purpose of this section of the work was to test whether the reported high variability in egg production was due to differences in the reproductive capacity of individuals or a consequence of the egg collection methods that have been employed previously. The final experiment (experiment V) quantified the variation in egg production both within and between pairs of fathead minnow over a period of 42 days. This is the time period recommended for the fish reproduction test (Gale and Buynak, 1982; Harries et al., 2000). The data derived from these experiments were used to inform on the appropriateness of the fathead minnow as a test organism for assessing the effects of chemicals on reproductive capacity in the 42-day fathead minnow reproduction test.

2. Animals, materials and methods

2.1. General experimental conditions

2.1.1. Test organisms

Details regarding the supply of the fathead minnows for each investigation are provided in the relevant sections. All fish were held for a minimum of 3 months in the husbandry unit at AstraZeneca's Brixham Environmental Laboratory, Devon, UK, prior to the initiation of any test procedures, to ensure that they were free from disease and at the stage of reproductive development required for the pair-breeding studies.

A minimum of 2 weeks prior to the start of each study, sexually mature males and females (determined by the development of secondary sexual characteristics) were separated to prevent any spawning activity and acclimated to the test conditions. During all acclimations and experiments, the fish were maintained in de-chlorinated water at 25.0 ± 1 °C, with a 16 h light:8 h dark photoperiod, with 20 min dawn and dusk transition periods. Fish were fed frozen brine shrimp (Tropical Marine Centre, Hertfordshire, UK), twice daily (approximately 0.9 g/feed tank), and Ecostart 17 pelleted fish food (approximately 0.1 g/feed tank) BIOMAR, Houghton Springs Fish Farm, Dorset, UK), once daily. Uneaten food was siphoned from the tanks once daily.

2.1.2. Water quality

The supply of de-chlorinated water to the laboratory dosing system was monitored daily for conductivity, weekly for alkalinity, hardness and free chlorine, and monthly for total ammonia. Throughout all studies the conductivity of the test water ranged from 206 to 282 μS/cm, alkalinity ranged from 20.0 to 30.6 mg/L, the hardness from 38.3 to 50.0 mg/L (as $CaCO_3$). Free chlorine remained below 2.0 μg/L and ammonia (as $N\text{-}NH_3$) was below 10 μg/L. Dissolved oxygen concentrations and pH levels were determined in the individual tanks on days 0 and 1 and then twice weekly throughout the exposure period. In all experiments, the dissolved oxygen concentration remained >70% of the air saturation value and pH values ranged from 6.9 to 8.0. Water temperatures were monitored constantly throughout the exposure period and ranged between 24.3 and 25.2 °C.

In all experiments fish were held under flow-through conditions. The de-chlorinated water was dosed into each tank, via a

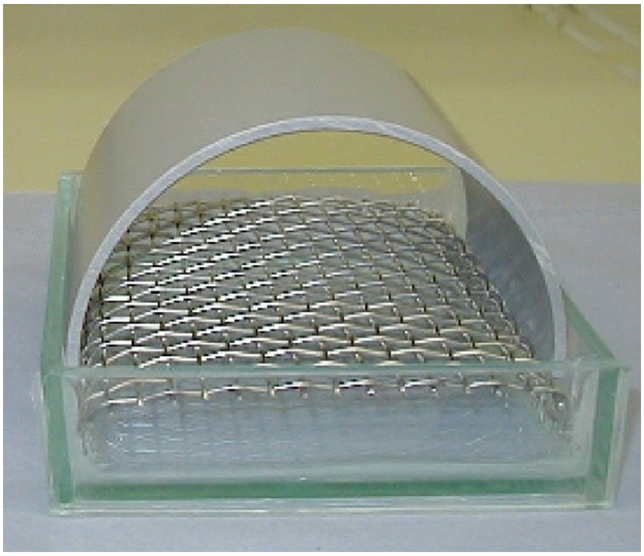

Fig. 1. Picture of the modified egg collection system, showing the commonly used inverted U-shaped PVC tile that has been placed above a screened egg collection tray.

gravity fed system, at a nominal rate of 80 mL/min. This flow rate provided a 95% replacement time of approximately 6 h. Flow rates were checked at least once per week and remained within 20% of nominal.

2.1.3. *Test apparatus*

The test vessels used for the adult pair-breeding studies had a working volume of 12 L (305 mm × 150 mm × 355 mm; length × width × depth) and were constructed of glass, with a minimum of other materials (silicon rubber tubing and adhesive) in contact with the test solutions. Screens were placed around the sides of all test vessels to prevent fish interacting with those in neighbouring tanks, and to minimise disturbance due to operator movements. The spawning substrates consisted of a tile (80 mm length of 110 mm diameter PVC half guttering; US EPA, 1987) placed above a screened collection (Sc) tray (Fig. 1). The Sc tray consisted of a rectangular glass tray, measuring 130 mm × 110 mm × 30 mm (length × width × depth) covered with 0.5 cm^2 stainless steel mesh (the mesh size allowed eggs to pass through to the collection vessel, while preventing the passage of the fish). In experiment I, a non-screened collection (N-Sc) tray was also used. The N-Sc tray consisted of a rectangular glass tray measuring 130 mm × 260 mm (length × width); the aim of this tray was to resemble the base of the tank and so, to compensate for the lack of sides to the tray, a larger piece of glass was used to ensure collection of all eggs. The height between the spawning tile and the surface of the Sc and N-Sc tray was between 60 and 70 mm. In all experiments, the spawning substrates were removed from the aquaria and checked for the presence of eggs, once daily at between 10:30 and 11:00 a.m., and the tiles and trays replaced with clean ones, regardless of the presence or absence of eggs.

The test vessels used for the hatching trials had a working volume of 9 L (305 mm × 205 mm × 210 mm; length × width × depth) and were constructed of glass, with a minimum of other materials (silicon rubber tubing and adhesive) in contact with the test solutions.

2.1.4. *Experimental design*

To initiate each test, males and females were selected at random from the holding tanks and placed as pairs into replicate test vessels containing a spawning substrate. The fish were acclimated to the spawning test conditions for a minimum of 7 days, before any detailed assessments of reproductive performance were made. After this initial acclimation period, daily assessments of the reproductive behaviour (observational only) and spawning activity (quantified assessments of egg number) for each pair were assessed.

2.2. *Experiment I*

The fathead minnows used in this experiment were supplied as juveniles (approximately 6 months old) by Aquatic Research Organisms (Hampton, USA) and held for a period of approximately 10 months at Brixham Environmental Laboratory, before starting the experiment. The body weights (as means ± S.E.M.) of the male and female fish at the start of the experiment were 6.67 ± 0.23 g ($n = 30$) and 2.82 ± 0.10 g ($n = 30$), respectively. A total of 40 pairs of fish were used in this experiment and these were divided randomly into four groups, according to the type of spawning substrate used, giving a total of 10 replicate pairs of fish per group. The first group was provided with a spawning tile and N-Sc tray, the second group, received a spawning tile and Sc tray. The third group of fish was initially provided with a spawning tile and N-Sc tray, then after a period of 14 days the N-Sc tray was replaced with a Sc tray. The fourth group was initially provided with a spawning tile and Sc tray, then after a period of 14 days the Sc tray was replaced with a N-Sc tray. As it has previously been observed, in both our laboratory (personal observations) and others (McMillan, 1972; Gale and Buynak, 1982; Harries et al., 2000), that eggs fall to the bottom of the tank during spawning, it was not felt appropriate to include a group without any form of tray collection system. After an acclimation period of 10 days, the number of eggs spawned by each pair of fish was determined daily for a total of 28 days.

Additional investigations were conducted to determine the viability of eggs in the collection tray, and these were compared with those adhered to the tile. When more than 50 eggs were counted, both on the spawning tile and the egg collection tray for an adult pair, embryos from the spawning tile were rinsed and collected into a Petri-dish containing de-chlorinated water. A total of 50 eggs were taken at random from the Petri-dish and transferred to replicate incubation cups (25 eggs/cup). This procedure was repeated for eggs taken from the corresponding N-Sc or Sc tray. The incubation cups were suspended from an oscillation unit into tanks receiving a continuous supply of de-chlorinated water, as described for the adult exposures. Embryo viability was assessed daily until hatch (4–6 days) and any dead embryos discarded to minimise the risk of fungal contamination. When less than 50 eggs were present, both on the spawning tile and egg collection tray, all eggs were removed from the spawning surfaces and discarded after counting with no assessment of viability.

2.3. Experiments II–IV

The fathead minnows used in experiment II were supplied as juveniles (age unknown) by Osage Catfisheries Inc. (Missouri, USA) and held for a period of approximately 3 months at Brixham Environmental Laboratory, before starting the experiment. The fathead minnows used in experiments III and IV were bred at Brixham Environmental Laboratory, and were approximately 5 and 8 months old, respectively, at the onset of each experiment.

A total of 36, 48 and 48 pairs of fish were used in experiments II, III and IV, respectively. The body weights (as means ± S.E.M.) of the male and female fish at the start of each experiment were 4.21 ± 0.13 and 2.21 ± 0.06 g, respectively, in experiment II, 4.47 ± 0.11 and 2.08 ± 0.08 g, respectively, in experiment III, and 5.52 ± 0.12 and 2.69 ± 0.11 g, respectively, in experiment IV. In each experiment, the number of eggs present on the PVC spawning tile and in the mesh-screened tray, was determined daily for each pair of fish, for a period of 24 days.

2.4. Experiment V

The fathead minnows used in experiment V were from mixed suppliers including Osage Catfisheries Inc. and Brixham Environmental Laboratory. All fish had been held at Brixham for a minimum of 3 months before starting the experiment. A total of 30 pairs of fish were used in the experiment and these varied in age from 5 ($n = 8$), 7 ($n = 8$) and 8 ($n = 8$) months to >8 months (exact age unknown; $n = 6$). Each pair of fish was provided with a PVC spawning tile and a mesh-screened egg collection tray.

The purpose of this experiment was to mimic the exposure duration used in the adult fish reproduction test (Harries et al., 2000; Ankley et al., 2001) and to determine whether the reproductive capacity of fathead minnow is comparable between the two study periods at the level of the individual. In the adult fish reproduction test, the rationale is to determine egg production over a pre-exposure period of 21 days and so establish the baseline egg production for each pair/group of fish. Exposure to the test chemical is normally then initiated and egg production monitored for a further 21 days. Egg numbers are subsequently compared for the two time periods for each individual pair/group of fish to determine the effect of the chemical on reproduction while controlling for the inherent variability in reproductive capacity between individuals. To enable such a comparison it is, therefore, important that the reproductive capacity of fathead minnow does not vary as a function of time. As it has previously been demonstrated that the initial spawning events for pairs of fathead minnow tend to be smaller than subsequent spawnings (Gale and Buynak, 1982) an initial acclimation period was included in this experiment to enable exclusion of these first spawning events. The pairs of fish were acclimated to the test conditions for 11 days, during which reproductive behaviour and spawning activity was monitored daily to confirm that each pair of fish were compatible and were actively spawning. After this initial acclimation period, accurate assessments of the number of eggs spawned by each pair of fish were conducted daily for a period of 42 days.

2.5. Statistical analyses

Within this manuscript, the reproductive capacity of the fish is expressed as egg batch size, total egg number, and egg production rate. Egg batch size is the average number of eggs spawned for the different spawnings for each pair of fish, total egg number is the sum of all eggs spawned per female over the total duration of an experiment and egg production rate refers to an average of the total number of eggs spawned per female per day. This latter term enabled experiments of different durations to be compared through standardising the measurements against time. It was considered necessary to include all three methods of expressing reproductive capacity, within this work, to provide a means of comparing these datasets with those from other studies where these different measurements have been used. Irrespective of the method selected for expressing reproductive capacity, in all cases the results are expressed as mean ± standard error of the mean (S.E.M.).

All statistical analyses were performed using Minitab Version 14.1. Data meeting the assumptions of normality and homogeneity of variance were analysed using one-way analysis of variance (ANOVA) followed by a pair-wise multiple comparison procedure (Tukey-test). In experiment I, an all pair-wise multiple comparison procedure (Bonferroni *t*-test) was used. Data which failed to meet the assumptions of both normality and homogeneity of variance were analysed using a Kruskal–Wallis one-way analysis of variance on ranks, followed by a pair-wise multiple comparison procedure (Dunnett's method for equal sample sizes and Dunn's method for unequal sample sizes). In all experiments, strengths of association between pairs of variables were measured using the Pearson product movement correlation coefficient.

3. Results

In each experiment it was necessary to remove some pairs of fish from the experiment, to comply with local animal welfare regulations, because the females in the breeding pairs became unable to release their eggs. In such cases, the female exhibited clear signs of distress and the abdomen became very swollen. In some cases, it was also necessary to exclude additional pairs of fish from the data analyses because they spawned on the sides of the tank, in addition to, or occasionally instead of, on the spawning substrate potentially compromising the accuracy of egg number determinants. The results and statistics shown for experiment 1 are based on a final sample sizes of 8, 6, 6 and 7 adult pairs, for groups 1, 2, 3 and 4, respectively. The results and statistics shown for experiments II, III, IV and V are based on final sample sizes of 30, 37, 35 and 20 adult pairs, respectively.

3.1. Experiment I

All pairs of fish established regular spawning activity, with spawning intervals of between 2.7 and 14 days (mean 4.75, mode

Table 1
Mean (±S.E.M.) egg numbers (total egg production over 14 days and number of eggs per spawning [egg batch size]) for fathead minnow ($n = 8$, 6, 6 and 7 pairs in groups 1, 2, 3 and 4, respectively) provided with either a spawning tile placed above a non-screened (N-Sc) or screened (Sc) egg collection tray

Group	Type of tray	Total egg production		Egg batch size	
		Tile only	Tile + tray	Tile only	Tile + tray
1	N-Sc	724 ± 125	841 ± 113	241 ± 28	286 ± 31
	N-Sc	714 ± 141	837 ± 162	229 ± 43	266 ± 46
2	Sc	1304 ± 141	1542 ± 110	301 ± 21	362 ± 24
	Sc	1183 ± 174	1470 ± 137	297 ± 40	376 ± 40
3	N-Sc	1079 ± 85	1168 ± 351	232 ± 46	264 ± 38
	Sc	757 ± 173	1133 ± 176	203 ± 27	321 ± 19
4	Sc	775 ± 159	1039 ± 168	273 ± 38	371 ± 24
	N-Sc	763 ± 188	851 ± 223	296 ± 34	327 ± 37

Egg numbers counted on the spawning tile alone, and on the spawning tile together with an associated collection tray are shown in separate columns. For each group, the first rows of data represent the egg numbers for days 1–14 and the second rows of data represent the egg numbers for days 15–28.

4.3) for the individual pairs. Table 1 shows the mean total egg numbers and egg batch sizes over each 14 day collection period for each group of fish, based on the number of eggs collected from the spawning tile only and the number collected from the spawning tile and Sc or N-Sc tray. Mean (±S.E.M.) egg batch sizes, based on eggs collected from the spawning tiles only, ranged from 69 ± 36 up to 484 ± 109 eggs/spawn, with an overall mean spawning batch size of 258 ± 13 egg/spawn (for all pairs). Total egg production over a period of 14 days varied from 130 for one pair of fish up to 2547 for another, with an overall mean of 893 ± 64 (equivalent to an egg production rate of 63.8 ± 4.94 eggs/female day). No significant differences were observed in egg batch size or total egg number between each of the four groups either within, or between, the two 14 days monitoring periods ($P > 0.05$), based on the tile only egg collection data.

Inclusion of eggs collected from the N-Sc tray increased the mean size of the spawn by 11% to 286 ± 20 eggs/spawn (range between 93 ± 30 and 525 ± 127 eggs/spawn for the individual pairs) and the total number of eggs (over 14 days) by 10% to 910 ± 102 eggs (the range was between 165 and 2553 eggs for the individual pairs). The corresponding rate of egg production increased to 65.0 ± 7.30 eggs/female day. The apparent higher estimates in egg number using the N-Sc, however, were not significantly different from those obtained using the estimates of egg number for the tiles only. Inclusion of eggs collected from the Sc tray, however, significantly increased both egg batch size estimates ($P < 0.01$) and total egg number ($P < 0.05$), compared with estimates for the tile only. Estimates of egg number using the Sc tray increased the egg batch determinants by 39%, to an overall mean of 358 ± 14 eggs/spawn (range between 254 ± 25 and 520 ± 90 eggs/spawn for the individual pairs) and the total number of eggs in 14 days by 44%, to 1286 ± 84 eggs (range between 307 and 1944 eggs). The corresponding rate of egg production was 91.8 ± 5.98 eggs/female day. In addition to a higher egg production, inclusion of eggs in the Sc trays also reduced the variation in egg batch size ($P < 0.05$), with a decrease in the coefficient of variation (CV) from 36% (tile only data) to 19% (tile and Sc tray combined). A reduction in the CV for total egg number over a period of 14 days, from 57% (tile only) to 33% (tile and Sc tray), was also observed but this was not found to be statistically significant. In contrast, with the use of the Sc tray, use of the N-Sc tray did not affect the variability of the estimates of egg number compared with estimates from the tile only ($P > 0.05$).

The hatching successes of embryos collected from both the spawning tile and tray were compared. For this, embryos were collected within 6–8 h of spawning, i.e. the embryos were between the stages of early cleavage and morula. Embryos that were collected from the spawning tiles hatched within 4–5 days, with a mean survival to hatch of 85 ± 2.6%. Embryos collected from the spawning tray similarly hatched within 4–5 days, but their survival to hatch was lower than those collected from the tile (mean 63 ± 5.7%; $P < 0.05$). Most embryos that were left in the tray for 24 h post spawning did not develop beyond the tail free stage and mortality was greater than 80%. In contrast, embryos left on the spawning tile underwent normal embryonic development. When the eggs from the tray were cleaned thoroughly, by rinsing them in de-chlorinated water and removing any debris adhered to their surface, survival to hatch increased to more than 85% and did not differ from the hatching success of embryos collected from the spawning tile.

3.2. *Experiments II–IV*

In all three experiments, fish acclimated to the test conditions relatively quickly, and established regular spawning patterns. Spawning intervals varied between pairs of fish, from 2.4 to 4 days (mean 3.3, mode 3.0) in experiment II, from 2.4 to 8 days (mean 3.5, mode 3.4) in experiment III and from 2.4 to 6 days (mean 3.8, mode 3.4) in experiment IV. Mean egg batch sizes, based on numbers of eggs spawned on the tiles only, were 88 ± 12 (ranging from 5 ± 3 to 234 ± 57), 187 ± 15 (ranging from 30 ± 24 to 437 ± 27) and 230 ± 20 (ranging from 43 ± 12 to 588 ± 46) in experiments II, III and IV, respectively. Mean total egg numbers (over a 24-day period) were 639 ± 87 (range from 23 to 1871), 1394 ± 130 (range from 208 to 3057) and 1532 ± 134 (range from 171 to 3526) in experiments II, III and IV, respectively. Corresponding rates of egg production were 26.6 ± 3.61, 58.1 ± 5.41 and 63.8 ± 5.58 eggs/female day in experiments II, III and IV, respectively. Consistent with the results from experiment I, some individual pairs of fish did not succeed in attaching all of the eggs spawned to the under-surface of the spawning tile. The percentage of each batch of eggs deposited on the spawning tile varied between 2.1 ± 1.4% and 68.8 ± 15.2% (mean 28.2 ± 4.0%) for pairs of fish in experiment II, between 10.3 ± 4.9% and 90.1 ± 1.7% (mean 56.4 ± 3.3%) in experiment III and between 11.5 ± 3.4% and 96.9 ± 0.6% (mean 64.2 ± 3.7%) in experiment IV. Mean egg batch sizes were higher with the use of the Sc tray ($P < 0.05$) and were increased by 198% compared with the tile alone in experiment II (mean 263 ± 12 eggs/spawn; range 139 ± 13 to 394 ± 14), 59% in experiment III (mean 298 ± 12 eggs/spawn; range 156 ± 24 to 485 ± 31) and 47% in experiment IV (mean 339 ± 16 eggs/spawn; range 211 ± 20 to 672 ± 53). Similarly, use of the Sc tray resulted in an increased mean total egg number (over a 24-day period; $P < 0.05$), by 202%, compared with the tile alone in experiment II (mean 1927 ± 99 eggs; range 934 to 3149), 56% in experiment III (mean 2170 ± 114 eggs; range 871 to 3448) and 46% in experiment IV (mean 2232 ± 108 eggs; range 928 to 4033). Corresponding rates of egg production were 80.3 ± 4.13, 90.4 ± 4.74 and 93.0 ± 4.48 eggs/female day in experiments II, III and IV, respectively. In addition to increased estimates of egg production for each pair of fish, use of the Sc tray was also observed to result in a reduced variation in egg number estimates between the individual pairs of fish. The CV for egg batch sizes was reduced from 75% to 24% in experiment II, from 50% to 25% in experiment III and from 52% to 28% in experiment IV. The CV for total egg numbers (over the 24-day period) between individual pairs of fish was also reduced with the use of the Sc tray from 74% to 28% in experiment II, from 57% to 32% in experiment III and from 52% to 29% in experiment IV. In addition, reductions in the CV were also observed in egg batch size between separate spawning events for each individual pair of fish. The mean within-pair variation in egg batch size was reduced from 100% (range 58 to 176%) to 28% (range 10 to 63%) in experiment II, from 65% (range 17 to 211%) to 28% (range 7 to 54%) in experiment III and from 50% (range 10 to 157%) to 26% (range 9 to 58%) in experiment IV.

In every experiment, pairs of fish that spawned a large number of eggs in each spawning event were also more successful at depositing a higher percentage of their eggs onto the spawning tile ($P < 0.05$, experiment II; $P < 0.01$, experiments II and IV; Fig. 2). It was noted, however, that in experiments, II–IV, egg batch size increased as a function of time, with a batch size in the final spawning (over a 24-day period) almost twice that of the first spawning ($P < 0.01$). The mean egg batch size, over a period of 24 days, increased from 169 ± 18 to 340 ± 20 in experiment II, from 175 ± 30 to 325 ± 23 in experiment III and from 236 ± 25 to 340 ± 32 in experiment IV. It is possible that the relationship observed between the percent of eggs on the spawning tile and egg batch size was an indirect result of the pairs of fish becoming more proficient at depositing their eggs on the spawning tile with time. The results from experiments II and III appear to support this with the percentage of eggs deposited on the spawning tile increasing from 7% to 45%, in experiment II ($P < 0.01$) and from 40% to 92% in experiment III ($P < 0.01$) in the successive batches over the 24-day monitoring period. In experiment IV, however, there was no such evidence that the individual pairs of fish improved in their ability to deposit their eggs on the spawning tile with time ($P > 0.05$).

In experiments II–IV, inclusive, there was no evidence that the percentage of eggs deposited on the spawning tile was influenced by the body weight of either the male or female ($P > 0.05$) fathead minnow in the breeding pair, or the male:female body weight ratio.

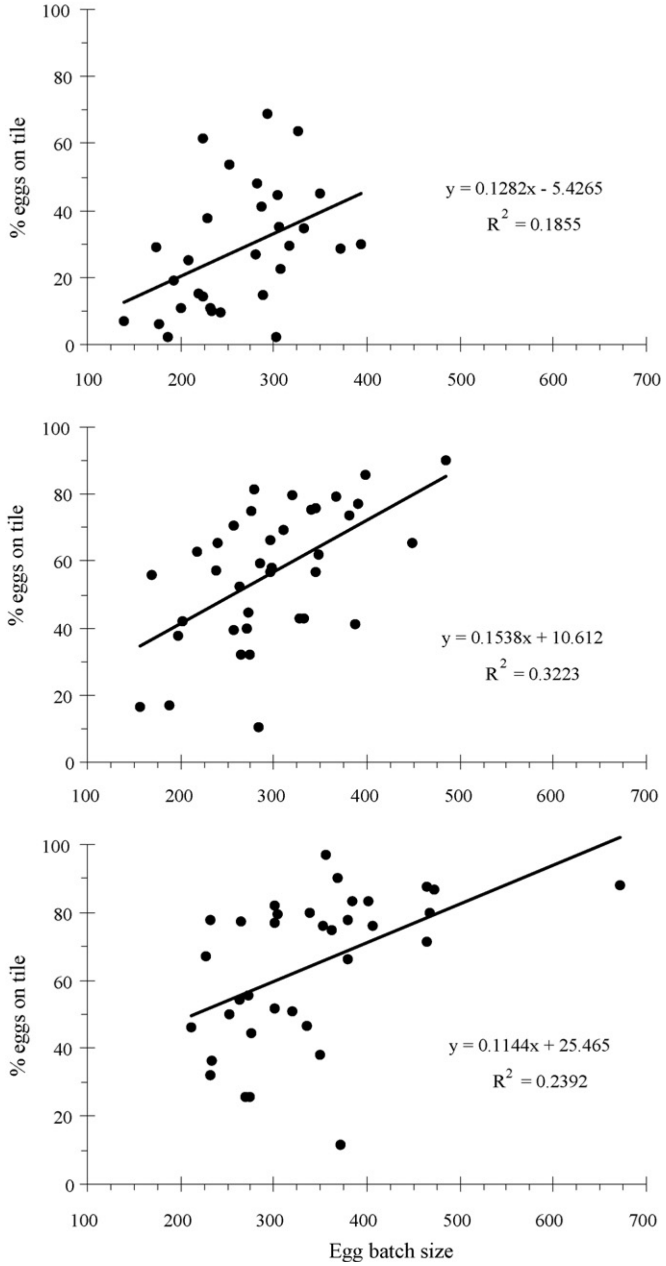

Fig. 2. Assessment of the relationship between the percentage of eggs in each spawn that were attached to the spawning tile and the size of the egg batch spawned in experiments II (A), III (B) and IV (C).

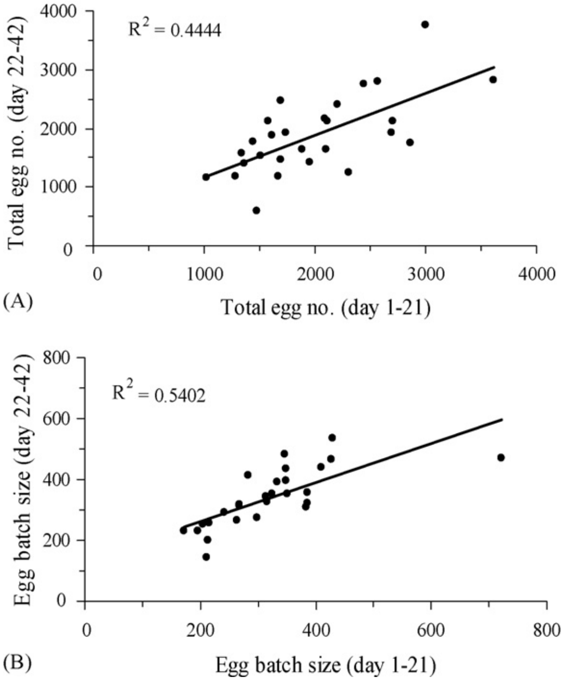

Fig. 3. Demonstration of the consistency in egg production (A: total egg number; B: egg batch size) for individual pairs of fathead minnow over two consecutive 21-day periods of study (experiment V).

3.3. Experiment V

The fish acclimated to the test conditions quickly, and established regular spawning patterns within 7 days. Spawning intervals varied between pairs of fish, from 3.0 to 4.2 days (mean 3.4, mode 3.0) during the first 21 days, and from 2.6 to 7 days (mean 4.0, mode 3.5) during days 22–42. Mean egg batch sizes, based on numbers of eggs spawned on the tiles only, were 321 ± 21 eggs/spawn (ranging from 171 to 722 eggs/spawn) for the first 21 days, and 339 ± 18 eggs/spawn (ranging from 145 to 536 eggs/spawn) for days 22–42. Mean total egg numbers (over a 21-day period) were 1999 ± 118 (range from 1027 to 3610) for the first 21 days, and 1882 ± 128 (range from 581 to 3755) for days 22–42. Corresponding mean egg production rates were 95.2 ± 5.6 eggs/female day for the first 21 days, and 89.6 ± 6.11 eggs/female day for days 22–42. Comparison of the reproductive performance (total egg number and egg batch size) demonstrated a high consistency between the two 21-day study periods for the individual pairs of fish (total egg number, $R^2 = 0.4444$, $P < 0.01$; egg batch size, $R^2 = 0.5402$, $P < 0.01$; Fig. 3). Mean coefficient of variations, based on data collected for the individual pairs of fish, for total egg production and egg batch size were 15.8 ± 2.6% (range from 0.5% to 61.7%) and 12.0 ± 1.5% (range from 0.5% to 29.7%), respectively.

4. Discussion

In these investigations comprehensive estimates of egg number were made for adult pair-breeding fathead minnow, held under controlled experimental conditions. In all experiments fish acclimated quickly to the test conditions and produced batches of eggs every 3.3–4.8 days. These spawning frequencies are consistent with those reported in the literature (Gale and Buynak, 1982; Harries et al., 2000; Ankley et al., 2001; Jensen et al., 2001). Estimates of egg number were highly consistent between each of the five experiments reported, in this study, with mean rates of egg production of 80 ± 4.1 to 93 ± 5.4 eggs/female day. This high consistency in the number of eggs spawned by fathead minnow pairs, across experiments, and consequently between different stocks of fish, however, contrasts with the reported literature where egg production rates range from 2.5 eggs/female day (Parrott and Blunt, 2005) to 112 eggs/female day (Harries et al., 2000). The high variation in estimates of egg production between different laboratories may relate, at least in part, to differences in the egg collection strategies used. Supporting this, in the two other laboratories that used an egg collection tray in association with the spawning tile, rates of egg production were comparable to those reported here (between 43 and 112 eggs/female day; Gale and Buynak, 1982; Harries et al., 2000). The lower egg production rates reported in the literature (between 2.5 and 26 eggs/female day; Ankley et al., 2001; Länge et al., 2001; Jensen et al., 2001, 2004; Parrott and Blunt, 2005) have been derived from laboratories that did not use a screened collection tray with the standard inverted U-shaped PVC spawning tile, to collect the eggs spawned (and therefore do not include eggs that have not attached to the spawning tile). In our investigations, when eggs present on the spawning tile only were used for determination of egg numbers, the egg production rates in each experiment were between 1.5- and 3-fold lower than the assessments that included eggs on both the tile and in the spawning tray. These results show that a significant proportion of the eggs spawned do not attach to the PVC spawning tile and that the inclusion of an egg collection tray in the study design increases estimates of the number of eggs spawned by fathead minnow.

Use of the screened egg collection tray had its most significant value in reducing the variability in estimates of egg number between pairs of fish within each experiment. The mean coefficient of variation (CV) in total egg numbers between the individual pairs of fish in each experiment, II–IV inclusive, was reduced more than two-fold to between 29% and 32% through use of the screened egg collection tray. This implies that some of the variability in estimates of egg number, between pairs of fish (when estimates were derived from egg collections using the PVC tile only), resulted from differences in the ability of the pairs of fish to attach their eggs to the spawning tile. Use of the screened egg collection tray also reduced the variability in estimates of egg number between the spawning events, for each individual pair of fish, by more than two-fold, to between 26% and 28%. This further implies that while some pairs of fish were consistent in the proportion of eggs spawned that they were able to attach to the spawning tile, most pairs were more variable. The use of the screened egg collection tray ensures that the inter- and intra-pair variation in attachment of eggs to the spawning tile does not impact on estimates of the number of eggs spawned by pairs of fathead minnow. This approach also improved the statistical power of the reproduction test. As an example, when total egg production was compared between groups of fish ($n = 8$

pairs) using ANOVA, the statistical power of the test required to detect a 40% decrease in reproduction increases from <0.3 (tile only) to >0.7 (when the eggs from both the spawning tile and the egg collection tray were included in the analyses). To maintain this level of statistical power with egg numbers collected from the spawning tile only, the number of replicate pairs assigned to each treatment group would need to be increased from 8 to 24 pairs. This, of course, has considerable ethical (fish usage), practical, and cost implications. A further illustration of the impact of the use of the screened egg collection tray on the variability in the estimates of egg production is demonstrated by the reduction in the inter-experiment CV from 53% (based on total egg numbers determined using the tile only) to 7% when eggs from both the tile and the screened tray were included in the assessments. This shows that the number of eggs spawned by pairs of adult fathead minnow is remarkably consistent between stocks of fish, irrespective of their original source, age or body size. The data presented would strongly advocate the uptake of the revised test design that includes use of a screened egg collection tray system for the adult reproduction test with fathead minnows.

The use of a non-screened egg collection tray (experiment I), in contrast with the use of the screened egg collection tray, was not an effective means of collecting the eggs that did not adhere to the tile. Although use of the non-screened tray resulted in higher estimates of egg number, variation in egg batch sizes and total egg numbers between individual pairs of fish was not reduced. It is most probable that use of the meshed screen over the collection tray prevents the adult fish foraging on the eggs/embryos in the tray and so provides a more accurate representation of the number of eggs failing to adhere to the spawning tile (McMillan and Smith, 1974; Harries et al., 2000).

It is not known why some pairs of fish were more successful than others in attaching their eggs to the spawning tile. Visual observation of the eggs present in the collection tray indicated that they had been fertilised and if removed from the tray these embryos continued to develop successfully. Attachment of the embryos to the tile is therefore unlikely to be affected by their viability. During the spawning act, male fathead minnows have to lift and press the females' ventral surface against the underside of the spawning object to stimulate the simultaneous release of eggs and sperm (McMillan, 1972). The ability of the male to enable the successful deposition of the eggs onto the spawning tile could, therefore, conceivably be influenced by the body size of the male and/or female or the ratio of the size of the male:female. In our studies, however, there was no statistical association between body size or body size ratio between the sexes and the proportion of eggs deposited on the tile. It was observed, however, that pairs of fish that spawned more eggs per spawn were more successful at depositing a higher percentage of their eggs on the spawning tile. Males and female teleosts produce and release pheromones to synchronise reproductive activities (reviewed in Stacey, 1983). It is possible that the more fecund females produced and released larger quantities of these pheromones resulting in an increased reproductive effort by the males and so a greater success rate in attaching the eggs to the tile. A further possible explanation is that the pairs of fish became more proficient at depositing their eggs on the spawning tile with time (and practice). The results from experiments II and III would support this with the percent of each batch of eggs successfully deposited on the spawning tile increasing from 7% to 45%, in experiment II and from 40% to 92% in experiment III for the successive spawnings over the 24-day monitoring period. In experiment IV, there was no evidence of such a relationship, however, these fish were highly successful at depositing their eggs on the spawning tiles from the onset of spawning activity.

One of the major advantages of the reproduction assay is that egg numbers for the fish assigned to each treatment group can be established for the experimental conditions employed prior to contaminant exposure. For determining possible effects on reproductive activity, it is the variability in egg number as a function of time for the individuals assigned to each treatment group, rather than the variability between treatment groups, that is arguably of greater importance. A statistical relationship was observed between egg batch size and time in experiments II–IV inclusive. In these experiments the size of the spawn increased with time and the final spawn (over a 24-day period) was almost twice the size of the first spawn. Further analysis demonstrated that this relationship was the consequence of an increase in the number of eggs per spawn during the first spawning events. This is consistent with the observations of Gale and Buynak (1982) who also found that the first spawning tended to be smaller. Exclusion of these initial spawning events in investigation V, through acclimating the pairs to the test conditions for a period of 10 days, resulted in a high statistical correlation between the two consecutive 21-day assessment periods for both total egg number and mean egg batch size for the individual pairs of fish. This demonstrates that individual fathead minnow pairs are consistent in their egg production over a 42-day study period. Reproductive performance for the two consecutive 21-day periods (presented as a ratio of the total number eggs spawned during the second 21-day study period divided by the total number eggs spawned during the first 21-day study period) was between 0.82 ± 0.10 and 1.07 ± 0.10 (CV values were between 19.3% and 29%) for the four groups of fish. Thus there was no general trend for reproductive capacity to increase or decrease over an extended period of study, further illustrating that the egg production in the fathead minnow, in the context of the experimental conditions described, is remarkably consistent over time. Although individual pairs of fish did differ in their reproductive capacity, this reproduction test design enables variability to be addressed through calculating reproductive performance ratios to normalise the variability. Alternatively, statistical techniques can be employed that enable the baseline fecundity for each treatment group to be included in the analysis, for example analysis of covariance (ANCOVA).

The results of these investigations demonstrate that egg number in the fathead minnow is a highly robust endpoint for investigating the effects of chemicals on reproductive function provided that the proposed assay design includes both a spawning tile and a screened tray for the collection of eggs not adhering to the spawning tile.

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