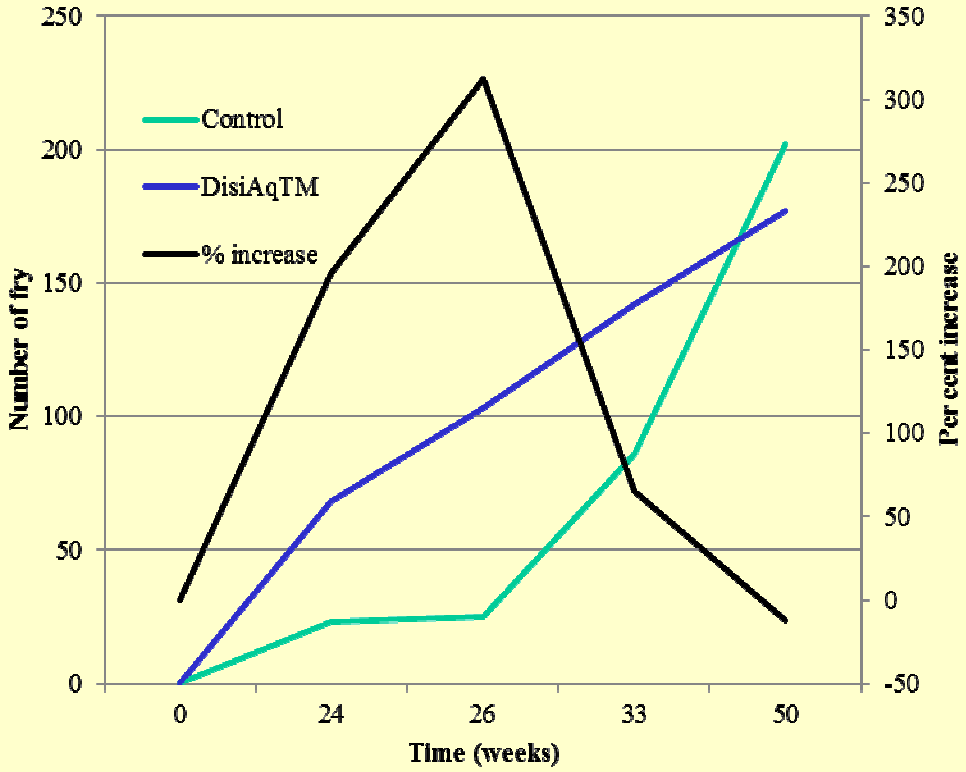
Guppies breed more quickly at London Zoo, UK

Week	Control (untreated)	DisiAq
0*	0	0
24	23	68
26	25	103
33	86	142

*i.e. immediately prior to study commencement in 2010

Increased fry production by guppies (*Poecilia reticulata*) exposed to DisiAq® via tank water, versus unexposed guppies. Note: tank microbes unaffected. See next slide for protocol and the slides after that for full experimental details.

Guppies (same experiment)



Experimental method (50 weeks)

- At the point of sexual dimorphism male and female individuals were separated
- At T0 males (10) and females (10) were placed in two 30 L aquariums
- DisiAq was administered on Mondays, Wednesdays and Fridays = 15 doses over five weeks
- Fry were counted at weeks 24, 26, 33 and 50 weeks
- The carrying capacity of the tanks was approached towards the end of the experiment. This impacted the test fish first, being by this time bigger on average than controls, blunting their population growth

Guppies (same experiment – in more detail)

Materials & Methods

Tank set up

A pre-experiment was conducted during the period when the fish tanks were being conditioned for the experiment proper. The issue was the number of species in the experimental system and the question of direct and indirect effects. A species of nematode worm (*C. elegans*) in studies with DisiAq had been fed dead bacteria rather than their normal diet of live bacteria to simplify the experiments down to a single live species. This was not practical for guppies, as a biofilter was required in each tank, for water purification purposes. The presence of an operational biofilter in the test and control fish tanks might have rendered interpretation problematic: would any observed changes be due to a direct effect on the fish or an indirect effect via the microbial population of the biofilter? Newly set up aquaria undergo a period of instability in the first several weeks with nitrogenous wastes building up in concentration until bacteria that utilise them become established in the tank. The filter in an aquarium provides a matrix for heterotrophic bacteria to colonise. These bacteria convert fish wastes from ammonia to nitrite and eventually to nitrate. Two identical aquaria were each filled with 30L of aged tap water. A new air-driven biological sponge filter was added to each tank and ammonium chloride was administered at 1mg per L per day to provide a food source of ammonia for the bacteria to establish themselves.

Fish study

The founder population of the guppies at the Zoological Society of London at Regent's Park, London, originated from a collection at the Pitch Lake in Trinidad in the mid-1990s. For the purposes of the trial 100 juveniles were selected at random from the main guppy colony and isolated in a separate 30L aquarium for maturation. Within two months sexual dimorphism was observed and the fish were divided into separate populations of males and females in order to prevent reproduction prior to the start of the study. Female guppies have the ability to store sperm packets in the folds of the oviduct, so a single mating can produce several broods over time. For this reason virgin status was necessary for the females to avoid starting the trial with fish that were already gravid, as the primary end-point of the study was number of offspring produced (fecundity).

Two glass aquaria of 30L each were filled with aged tap water and one AquaZoo air-driven sponge filter to provide biological filtration. A small amount of Java moss, *Vesicularia dubyana*, was added to each tank to provide cover for the fish, notably the fry, which might otherwise be subject to cannibalism. After five weeks the fish were old enough for sexes to be distinguished in the maturation tank and ten males and ten females were transferred to a DisiAq treatment tank and a control tank (i.e. DisiAq-free), in a randomized fashion. The two tanks were placed 2m off the floor on a shelf to minimise disturbance during the study. The fish were fed on a mixed diet of newly hatched *Artemia salinas* nauplii, Aquarian brand vegetable flake food, frozen Daphnia and cylops. Food was provided twice daily at 9am and 3pm.

The water quality parameters were tested weekly throughout the experiment using a Palintest photometer for pH, ammonia, nitrite and alkalinity. Temperature was measured daily using a glass thermometer and nitrate was measured weekly using a Salifert test kit.

DisiAq was added to the water of the test tank in solution from pre-dispensed vials, according to the supplier's instructions. (Controls received dosing vehicle only.) The first dose was administered during Week 1 (January 2010), then doses were given every other day for a total of 15 doses (including weekends) over a month. In prior nematode (*C. elegans*) studies it was found expedient to move the founding population of adults into fresh medium each day to facilitate counting and monitoring. With daily DisiAq administration to the worms, this meant that dose levels never exceeded the supplier's recommendation. For the guppies, alternate-day dosing reduced the likelihood of DisiAq accumulating above the recommended level, during the beta test phase of the product and its benign status not yet fully established. The test fish were observed immediately after DisiAq administration to check for signs of distress and any physical and behavioural abnormalities, with the control fish as comparators.

Continued…

Guppies – continued

The fry were counted during Week 24, 26 and 33, with a final count during Week 50. The size distribution of the fish populations were established during Week 26 with individuals measured to the nearest millimetre total length and categorized observationally as male, female or juvenile, according to their sexually dimorphic state or lack thereof (juveniles). At Week 33 the fish were moved from their original 30 L tanks to 50 L tanks for continued monitoring. This followed concern about the carrying capacity of the test tank and the potentially suppressive effect of this on fish behaviour.

Results

Tank set up

There was no discernable difference in the concentrations of either ammonia or nitrate in the two tanks in the biofilter pre-experiment. DisiAq cannot therefore be said to have changed the rate at which beneficial bacteria had become established. After four weeks the pre-experiment was terminated, with no difference between the test and control tanks in terms of water chemistry (data not shown).

Fish study

There were no mortalities during the study and at no point did the fish exhibit any signs of distress or ill health or show any physical abnormalities. Normal behaviour was observed, including feeding, courting, sparring and chasing. Water quality remained consistent throughout the trial and DisiAq did not affect water chemistry in any discernable way. The water quality data for the two tanks were essentially the same (Table 1).

Value	Control	DisiAq
NH4	0.03mg/l	0.03mg/l
NO2	0.01mg/l	0mg/l
NO3	5mg/l	5mg/l
pH	7.2	7.4
Temperature	23C	23C
Alkalinity	95	100

Table 1. Fish tank chemistry

Week	Control	DisiAq	DisiAq as % Control
0*	0	0	0
24	23	68	295
26	25	103	412
33	86	142	165
50	202	177	88

Table 2. Fry production by 20 guppies, DisiAq-exposed and unexposed (Control)
*i.e. immediately prior to study commencement in January 2010.

Fry production is shown in Table 2. DisiAq treatment had the effect of advancing the production of offspring, such that by Week 50 there were over fourfold as many fry in the test tank as in the control tank. Yet by the termination of the study at Week 50, controls numbers had surpassed those in the test tank. Including the 20 founding adults, the control tank had 222 fish at termination, the test tank 197. (Note: All data were included in the analysis; no outliers were excluded.)

Continued…

Guppies – continued

The size distribution of the fish populations at Week 26 showed a ‘left shift’, indicating many more fry in the test tank than the control tank (data not shown). Observation at termination (Week 50) revealed that there were far more large fish in the test tank than in the ultimately more populous control tank. This indicates that had the study accommodated another cycle of reproduction the number of test guppies would have exceeded once more the number of control fish.

After 6 months there were four times as many guppy fish fry in the DisiAq tank (right) than in the control tank (left).

Discussion

DisiAq given to guppies via their tank water was associated with accelerated fecundation, such that by the half-year mark there were over four times as many fry in the test tank as in the control tank. It is intriguing that merely adding DisiAq to the aqueous environment of fish should alter fecundity. To simplify interpretations, the nematodes in a prior experiment had been fed dead bacteria. The biofilter in the fish tanks could not be sterilised, but a pre-experiment involving biofilters without fish present indicated that biofilter microorganisms seem to be unaffected by DisiAq. DisiAq is easily administered in the manner of a supplement via tank water. Its mode of action is unknown, but could involve a subtle nutritional trigger effect on reproduction. The use of more drastic treatments in fish, to stimulate reproduction and increase fecundity, is common practice in aquaculture. Implants and injections of pituitary material for example have been used in both freshwater and marine species for food production. Even if desirable, implants or injections in guppies are infeasible due to their small size. With DisiAq simply added to the water, direct contact or absorption by the guppies could have taken place either via the gill membranes, through the skin or via ingestion with water.

The increase in fecundity in the test tank could have been due to improvements in either male or female reproductive potential. The supplement could increase egg size or number in female fish or increase the rate of ovulation. DisiAq could also increase male sperm productivity or motility. A third possibility is that mating frequency increased, with multiple matings taking place per female, thereby increasing the brood size or reducing the gestation period. (It is known that female guppies that engage in multiple matings exhibit an increased brood size and shorter gestation period.) This third possibility is unlikely since behavioural observations did not indicate an increased period of courtship in the test tank versus controls. Behaviour remained consistent in each tank even immediately after the addition of the DisiAq with no marked increase in courtship in either tank. Therefore an improvement in egg or sperm condition or size or number is more likely. Further work is needed to define the mechanism of action of this benignly efficacious pro-fecundity formulation.

Catfish - breeding resumes

“ Our Tiffany catfish, *Rineloricaria teffeana,* ceased spawning for no apparent reason; none of the pairs were reproducing, which is unusual and hadn't happened before. The Tiffanies were moved to another system with improved water quality and although pre spawning activity commenced on the new system for several weeks, nothing else happened. The Tiffanies were then fed with DisiAq® infused food for 5 days, and 6 days later spawning commenced again without any other stimuli.”

Fish breeder, 6 September 2010

Tiger stingrays induced to mate

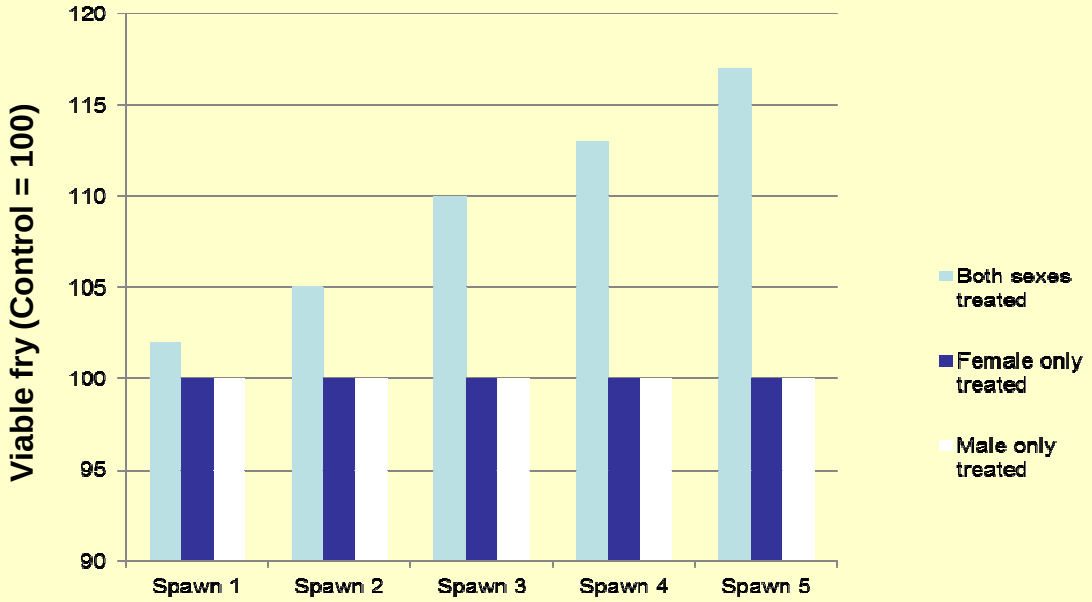
"I had collected tiger stingrays from South America until they were classified onto the CITIES listings as being endangered in the wild. Since they are rare their price was above that which most aquarists were prepared to pay for aquarium fish. Over a period of years I managed to get a compatible pair and kept them together in a large tank. I kept them in the parameters from their place of origin, but they just remained a happy compatible pair. Two years ago due to work pressure I bestowed them onto a friend who was very interested in attempting to breed them, he duplicated my protocol. Four weeks ago I fed the rays with dead whitebait (as usual) but for 5 days I added a DisiAq® capsule into the whitebait and sealed the whitebait afterwards. A week later I got a call from my friend who said the female had been attacked by the male. When I inspected the fish, she had all the signs of breeding damage as is common with all shark related species. The male was still attempting to mate with the female, so I had no option but to separate him from her, to prevent further damage within the confines of an aquarium. I do not believe there have been any other tank breeding successes with the tiger stingray, that I know of."

Marine Biologist, 23 August 2010

Note: In February 2011 the marine biologist reported that the female (initially 4-5 kg) had given birth to two healthy pups, after a gestation of 14 weeks. At mating the fish were ~3 years old, having been wild caught when "small but not newborn". Parents and offspring entered a private collection in Dubai. In November 2012 the marine biologist added, "Stingrays are very sensitive to toxins, and the tigers showed no adverse effects." In March 2013 this email message was received from the marine biologist: "I am led to believe the tiger stingrays I worked with and the pups have now been returned to South America and released." This is the first captive breeding success for DisiAq, involving release into the wild of newly bred individuals.

Angelfish 'his & hers' success

Experiment conducted at a commercial fish breeding establishment in the UK, 2011



Note: Reliable spawning seen only in 'Both sexes treated' group, with rising productivity; fewer, random spawnings in other test groups (as in controls); graph shows these as '100', i.e. normal, to overcome representational challenge
(see following slides for more details)

Summary

- **n = 180 (sexually mature, previously bred freshwater angelfish, *Pterophyllum scalare*)**
- **Duration of study ~4 months**
- **Multiple tanks, shared-water centralised system**
- **DisiAq® administered via food (five-day cycles starting T0 and after each spawning)**
- **Eggs counted, fry evaluated**
- **Four exposure modalities: untreated controls; both sexes treated; females only treated; males only treated**
- **There was a major positive effect in 'Both sexes' tank only, which was cumulative over successive spawning cycles; 17% more fertilized eggs, as well as quantity of resultant viable fry, by Spawn 5; and aggressive behaviour**

Experimenter's comment: "The control fish did not spontaneously spawn to any degree above what we would normally expect, in contrast to the both-sexes-treated fish, where there was a continuous noticeable increase in productivity after each cycle of DisiAq. Nor did the control fish remain paired, as they did in the both-sexes-treated fish. Both sexes had to be treated to show a benefit. Treating one sex only increased productivity (eggs, semen) for that sex alone, but the other sex couldn't keep up. Parents remained normal; eggs and fry normal: no evidence of toxicity."

Angelfish (same experiment – in more detail)

Experimenter's own account:

"The study lasted about four months in all. The test subjects were 180 angelfish (colour variants of the common freshwater angelfish *Pterophyllum scalare*), comprising 90 previously spawned pairs (i.e. adults of established fertility; selected brood stock fish of ~12 months age). During a rest period from breeding the fish were sectioned into groups, as follows, in a randomized fashion.

Tank 1 contained 60 (30 pairs resting fish)
Tank 2 contained another 60 (30 resting pairs) as control
Tank 3 contained 15 males
Tank 4 contained 15 males
Tank 5 contained 15 females
Tank 6 contained 15 females

A multiple tank arrangement was used, employing a very large centralised filtration system, so that the water parameters in each tank and for every fish were identical.

DisiAq was added to oil and vortexed with micro pellet food to ensure it was sealed within the food and wouldn't separate out into the water upon feeding and diffuse to the other tanks within the system. Post-filtration ozonisation of the water was used to ensure that if any DisiAq did escape in small quantities from the food, it could not be transferred to other tanks. The aim was that only specific fish would be exposed to the reproductive activator. The primary end-point was number of offspring (fecundity).

Tanks 1, 3 and 5 were fed DisiAq -infused food for the first meal everyday for 5 days (one cycle). No real change in behaviour was noted until between Day 7 and 10 when aggression and pre breeding behaviour commenced. Pairs that formed in Tank 1 were separated off into breeding tanks within the system; males from Tank 3 were added to the females in Tank 6, females from Tank 5 were added to males from Tank 4 to ensure that one sex only was "treated" in Tanks 6 and 4. As pairs formed in the tanks they were again separated out into individual breeding cubes, marked to indicate which batch the parent stock originated from. Eggs, once fertilised, were separated from the parents, and again marked accordingly for record purposes. They were raised artificially to check for deformities and retards within the batches. [Note that in nature retards and deformed fish die from natural selection and inability to obtain enough food. Raising artificially allows surplus food so that all subjects are nourished and grow.]

Continued...

Angelfish (in more detail, continued)

Results & Discussion

All the fish in Tank 1 paired and bred.
25% of the control fish bred (Tank 2). They produced a mean of 183 eggs over several cycles.
About 40% of the fish contained within Tanks 6 and 4 paired, where only one party had been treated, and eventually bred. Although fish from Tank 4 produced slightly above average quantities of eggs, the fertile number only remained as average, males not having had DisiAq treated food. Nothing but average results were obtained from Tank 6.

No further feeding of DisiAq food was given to the fish resulting from the uniting of Tanks 4 and 6. Most went back off cycle although some pairs spawned a couple more times.

Fish from Tank 1 were fed DisiAq infused food 2 days after their eggs had been removed from the previous spawning, in a 5 day cycle as previously, and again spawned - this time whilst receiving the enhanced food. Each spawning from the treated fish produced larger quantities of eggs with a greater portion being fertilised. The food/breeding cycles were repeated for 5 continuous sessions following the same protocol of giving treated food 2 days after egg removal.

Most of the Tank 1 fish went off cycle upon cessation of exposure, although some pairs continued spawning for several weeks afterwards. Due to the increased egg production – rising steadily to 17% more than controls by Spawn 5 – it was felt safest to stop treatment after Spawn 5 to allow the breeding stock to recover, unless nature felt otherwise.

To confirm, in the 'Both sexes treated' Tank 1 egg production AND eggs fertilised increased with each cycle. The percentage of deformities and retards did not increase with DisiAq treatment. Exposure to DisiAq caused breeding reproducibly; breeding in other tanks was random. There were no parental deaths during treatment with DisiAq and no signs of toxicity, physical or behavioural. Note that breeding stimulation was not caused by seasonal stimulae (e.g. massive water changes adjusting temperature and pH or similar, as all fish were systemised).

As near as possible this study complied with the ideal of a protocol in which only one parameter was changed. No breeding stimulus besides DisiAq was given to any specific fish; all shared the same water, within the same system. DisiAq performed well and safely in enhancing productivity, without exhausting the females."

Experimenter's comment after the angelfish study:

"DisiAq caused a tank of wild caught heckels (discus) to start courtship behaviour. These were sold before results were known, as they were in quarantine before being moved on. Wild caught discus are harder to get to spawn than the tank bred variants as they are seasonally induced spawners. This was an accident as someone used some of the angel-intended Love Bomb food on the wrong tank…"

Email 28th February 2013

Frog breeding success, Paignton Zoo, UK

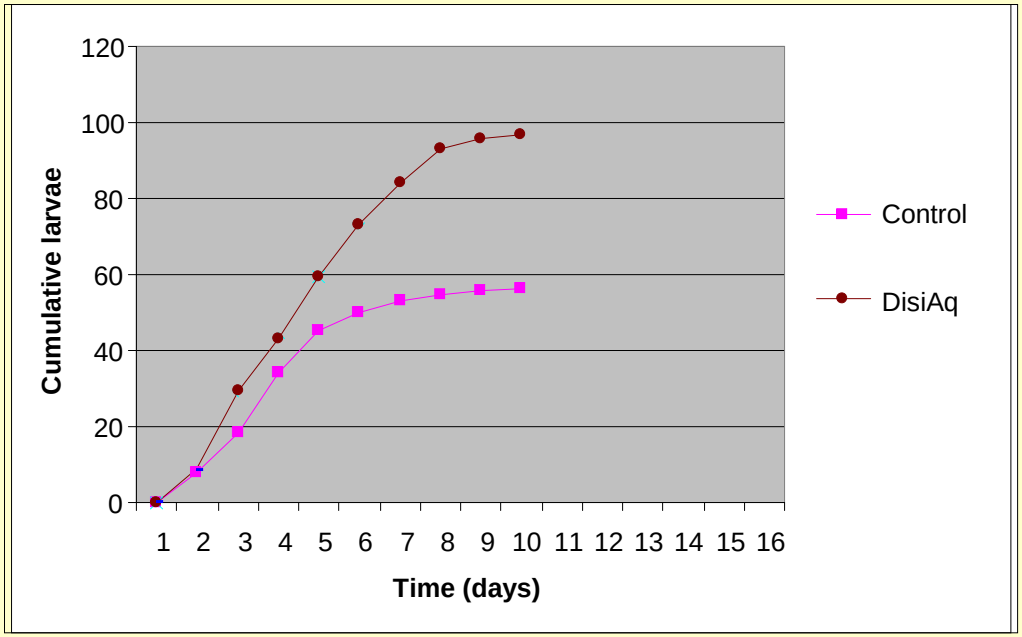
Email from: Mike Bungard, Curator of Lower Vertebrates & Invertebrates, Paignton Zoo, UK
Sent: 20 June 2012
Subject: Frogs exposed to DisiAq®

"The species bred was *Mantella ebenaui* [brown mantella] – a Malagasy species. I am not sure of the rate of success for this species in captivity as a whole, but I don't think it has bred before within zoos. It is a first for us with this species and it would be fairly safe to say that calling or breeding behaviour was close to non-existent before the trial."

Note: Single vivarium; DisiAq misted in water onto ~6 frogs daily, for five days; meticulous observations and record keeping; tadpoles pictured right.

Separately at Paignton Zoo, a group of splendid mantella frogs (*Mantella pulchra*, also from Madagascar) produced tadpoles after exposure to two five-day rounds of DisiAq, with a months-long interval between the rounds. The tadpoles appeared after a delay following the second exposure, indicating that the product can be regarded as mild, slow acting and as setting in train a process. The use of the product in any particular species or group of species should involve an open-minded experimental attitude. By January 2013 the two species of frogs at Paignton Zoo had produced between them 59 froglets. In terms of vegetation in the vivaria misted with DisiAq, Mike Bungard wrote this in March 2013: '…there have been no noticeable effects on plants, negative or positive.'

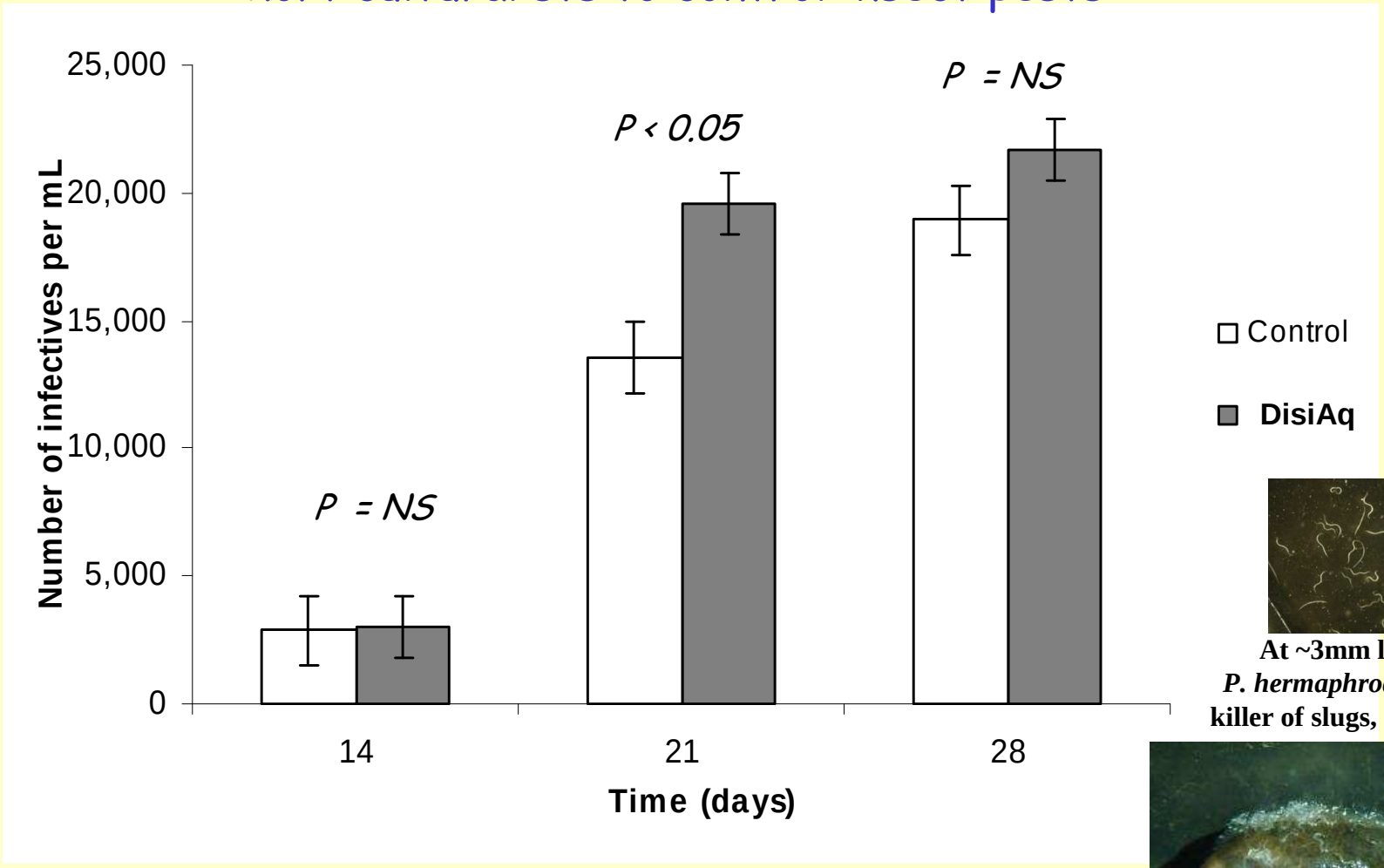
Nematode worm fecundity enhancement



The soil nematode *C. elegans* is <1mm in length. Experiment undertaken in staining blocks in a humid chamber with 10 worms per block.

DisiAq® increased fecundity in the nematode *Caenorhabditis elegans* to a significant extent (ANOVA, $p<0.02$). Fecundity was measured as 'cumulative larvae' (i.e. mean number of juveniles produced by four groups of 10 worms). The material was administered daily into the aqueous medium of the nematodes, throughout their lives. Controls were untreated. Bacteria were provided as food. There was no significant difference between the average lifespans of the two groups and no toxicity was observed among parents or offspring. The work was conducted by Dr Keith Davies, senior lecturer in molecular biology at the University of Hertfordshire, UK. The material tested was 'DisiAq Mark I', a development from 'Proto-DisiAq'. Further optimisation led to 'DisiAq Mark II', which was launched as a product in February 2013.

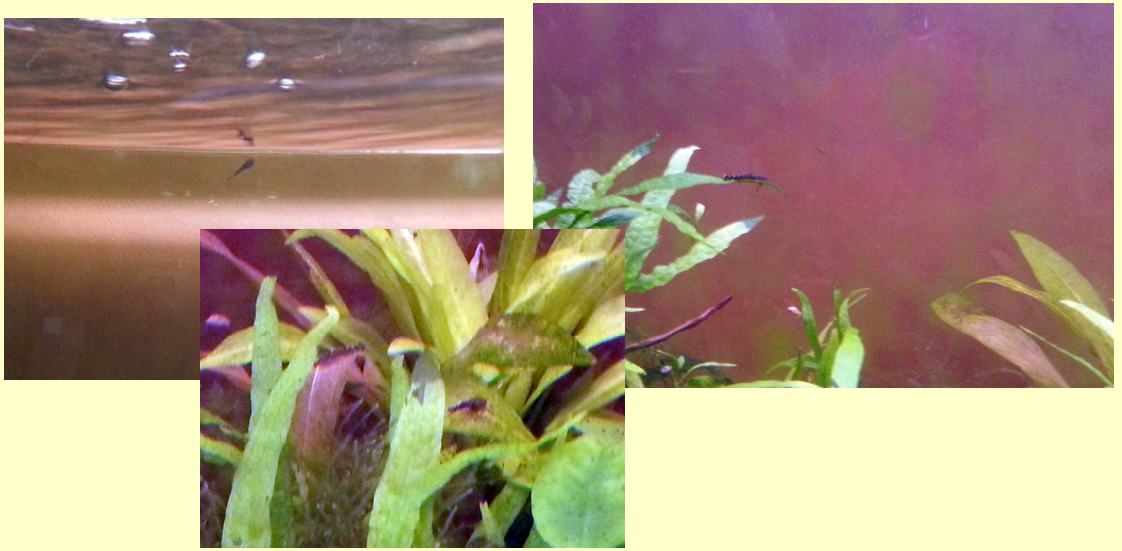
DisiAq® added to shaken cultures of *Phasmarhabditis hermaphrodita* by Becker Underwood, UK, a commercial breeder of entomopathogenic nematodes for use by gardeners and horticulturalists to control insect pests



At ~3mm long, *P. hermaphrodita* is a killer of slugs, as below

This is a notable result as DisiAq was administered daily via the worms' aqueous growth medium, which resembled liquid brick dust, at an industrial site. Time to peak production of the saleable 'infectives' was reduced by a week, i.e. a quarter, with numbers at this time point up by 45%.

Plecs & water snails propagate in a UK university hobbyist aquarium



From: Professor, UK university
Email sent: 17 August 2012
Subject: Plecs & water snails

"Breaking news: the snails are simply going to take over the world, starting from this tank here. Exponential reproduction. In the tank are a pair of Plecs [Bristlenose Plecostomus] and see attached, DisiAq® has kicked them into action and we have babies. Lots of them. Tricky to photo but these are fry looking like tadpoles sticking to the tank and leaves. Really exciting stuff here..."

Update, 03 Oct 2012: "The Plecs won't stop mating now, this stuff is brilliant when it works, just nailing how to make it work for different species is going to be the trick. They're on their 3rd batch of eggs." Note that Discus fish in the same tank did not breed and have subsequently come under suspicion of harbouring intestinal flagellate parasites. Treatment for this infestation initiated in 2013.

Update 16 Nov 2012: "The Plecs (tropical fish) have had three clutches of eggs all have been fine, other than the bigger fish have eaten most of them in the normal pyramid of life. Enough (a handful) have survived from each and are getting bigger and bigger by the week." [Note: The Plecs had not bred in the 8 months they had been in the tank prior to breeding and they were believed to be nulliparous. Administration via water had not worked, as the tank, at 400L had exceeded the recommended maximum of 125L. The product had then been administered via food, 0.5ml blood worms soaked in that day's liquid-activated Solid Components.