PESTSMART

Making and using female sex pheromone implants which attract mature male common carp

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"Together, create and apply solutions"

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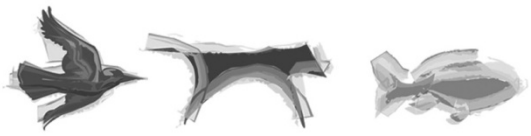

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Summary

The common carp, *Cyprinus carpio*, like many fish species, relies on pheromones (chemical signals that pass between members of the same species) to mediate reproductive success.

At least five sex pheromones have been characterized in this species, one of which is released by ovulated females and attracts spermiated, receptive males. This pheromone is comprised of prostaglandin F2α, its metabolites and other unknown polar body metabolites.

This manual describes an implant technology which can elicit release of this entire pheromone at extraordinarily high concentrations for a couple of weeks so that it can be used as bait to attract and trap sexually mature and receptive male carp.

1. Introduction to the common carp and its sex pheromones

1.1 The common carp and its pheromone system

The common carp (*Cyprinus carpio*; hereafter 'carp') is a large species of cyprinid fish from Eurasia which has been introduced across the globe and become super-abundant in North America and Australia (Koehn 2004; Bajer & Sorensen 2010). The carp is considered an 'environmental engineer' and has inflicted large-scale damage to shallow water ecosystems, largely through its habit of digging in the bottom for food and uprooting vegetation (Weber & Brown 2009). This species is especially difficult to control because it is both long-lived and highly fecund, resilient yet highly mobile, and seemingly clever enough to learn and avoid nets (Bajer *et al*. 2010; Thwaites *et al*. 2010). Although large scale removal of aggregating carp is possible using radio-tag implanted carp ('Judas fish') to reveal the location of the aggregations (Inland Fisheries Service 2009; Bajer *et al.* submitted), this technique cannot be applied at all locations because of bottom structures. Furthermore, it is not easy to remove the low numbers of carp it can leave behind. Targeted trapping might be best for this and it could be improved by using specific designs (Schwartz 1986), locations (Thwaites *et al*. 2010), and baits which employ either food or sexual odours (pheromones) (Sorensen & Stacey 2004). Pheromone induced trapping might also be useful to census populations of adult carp as part of integrated pest management schemes.

The carp and its relatives are known to rely heavily on sex pheromones, or chemical cues which pass between members of the same species (Sorensen & Stacey 2004), to mediate most aspects of their reproduction which is typically highly synchronized and intense, and therefore targetable (Stacey & Sorensen 2009). Indeed, laboratory studies have shown that both the carp and the goldfish, *Carassius auratus*, suffer from reproductive failure if their olfactory systems are occluded as they cannot identify mates (Stacey & Kyle 1983; Lim & Sorensen, unpublished results). Both male and female sexual pheromones have been identified in the carp which serve a variety of complex physiological and behavioural functions from synchronizing hormone levels to promoting ovarian and testicular final maturation to pre-spawning attraction (reviewed in detail by Stacey & Sorensen 2009). Because carp spawn at daybreak and their habitats are murky, sexual cues are very important to mediate behavioural interactions between males and females. Five sex pheromones have now been identified in the carp, and all are mixtures which include hormonal derivatives. These 'hormonal

pheromones' are detected with great specificity at low concentrations by mature carp (picomolar or grams in tens-to-hundreds of millions of litres of water) (Stacey & Sorensen 2009).

1.2 The female spawning pheromone

This document focuses on the female spawning pheromone which female carp release when ovulated and spawning and which we now know to be largely comprised of prostaglandin F2α (PGF2α) and its metabolites. Although F prostaglandins (a class of fatty acids) are produced in small quantities by most tissues of the body and serve various paracrine and auotocrine functions, F prostaglandins are also produced in great quantities as hormones by the oviduct of many fishes in which they stimulate follicular rupture and female sexual behaviour (Sorensen & Goetz 1993). In some cases the latter function is also linked with sex pheromone production as PGFs are generally rapidly cleared (Sorensen *et al.* 1998; Stacey & Sorensen 2009).

Ongoing studies in both the goldfish (Sorensen *et al.* 1986; 1988; 1989) and the carp (Lim & Sorensen, submitted) show that the female spawning pheromone cue is both potent (picomolar thresholds) and species-specific. Unlike the male sex pheromone, which is known to attract ovulated female carp, the key components of the female sex pheromone (i.e. the cue released by ovulated females) are relatively environmentally friendly (androgens partially comprise the male-derived sex pheromone), and its production can be stimulated through the use of implants (described here). Further, a key component and precursor, PGF2α (pharmaceutically known as dinoprost) can be relatively inexpensive to purchase, unlike the C21 steroids which comprise the pre-ovulatory female sex pheromone. A brief review of the female spawning pheromone follows.

Like most externally fertilizing teleost fishes, the process of ovulation and the onset of female spawning behaviour in the carps (i.e. goldfish, common carp and crucian carp [*Carassius carassius*]), appears to be triggered by massive surges in the production of circulating PGF2α (Sorensen & Goetz 1993; Stacey & Sorensen 2009). Female spawning behaviour (searching for and entering into floating vegetation for oviposition and tolerance of accompanying males) must coincide with ovulation because typically ovulated eggs are only fertile for a few hours. Conspecific males have evolved sophisticated means to detect and locate these sexually receptive females, which may only spawn a few days a year. A key to this process is the sex pheromone that ovulated females release which is primarily comprised of PGF2α and its metabolites.

Nanogram levels of PGFs are released by ovulated female carps at the precise onset of ovulation and terminates when they are spawned out (Sorensen, unpublished data). At least three PGFs products are released: PGF2α itself, and its immediate metabolites, 15-keto-PGF2α, and 13,14-dihydro-15keto-PGF2α (Lim & Sorensen, submitted). All are detected with great sensitivity and specificity by the carp and goldfish olfactory systems although in a sexually dimorphic manner (i.e. males are much more sensitive) (Sorensen *et al.* 1988; Irvine & Sorensen 1993; Sorensen & Goetz 1993). Interestingly, most of these products exit the female in the urine, the release of which the female controls and pulses to mark spawning substrate when males are present (Appelt & Sorensen 2007). Recent studies have also firmly established that full activity of PGF pheromone requires both the presence of PGFs and unknown polar body metabolites which strongly synergize the actions of the PGFs to enhance activity and give it a species-specific character (Lim & Sorensen, submitted). It is the need for this complex mixture that makes the use of implants critical because the donor fish used for the implants add the polar components to the pheromone, giving it the full activity required for field application.

The actions of the F prostaglandin-based female spawning pheromone are only partially understood. Nevertheless, studies clearly establish that the pheromone can attract male carp and the distance of activity will depend on concentration gradients and structure (Partridge *et al*. 1976; Sorensen *et al*. 1986; Lim & Sorensen submitted, unpublished results). In addition, this cue has been shown to stimulate sexual arousal in exposed mature males for at least 15 minutes during which time they will start to swim quickly and interact as they search for the cue. Behavioural responses are accompanied by sex-hormone surges which further enhance arousal and sensitivity to the pheromone (Sorensen *et al.* 1989). It is very important to note that only sexually mature and receptive male conspecifics respond to the spawning (PGF) pheromone (i.e. fish that have been exposed to female stimuli recently) (Lim & Sorensen, unpublished results). While females do not respond to their own odour directly, they will follow stimulated males to the source of the pheromone.

2. Pheromone implants and trapping

2.1 The concept

A sex pheromone might be useful in targeted trapping of carp for assessment and/or removal, especially in situations where relatively few carp remain. Of the many pheromonal cues identified, the female spawning pheromone is the most suitable for immediate proof-of-concept testing for reasons outlined above. Nevertheless, several challenges face those wanting to use it in the natural environment. First, it must be produced and added in super-normal concentrations as fully active (natural) mixtures are required to out-compete background stimuli. Second, it must be added in a way that produces concentration gradients the fish can follow. Third, trapping schemes are needed to effectively capture any carp that are attracted. Lastly, sex pheromones must be added at places and times when fully mature / sexually active males are present; their actions are highly specific and thus only effective during narrow windows of time. This document addresses the first challenge which we have found can be met through the use of implants.

Although production of the female spawning pheromone can be elicited simply by injecting PGF2α into female carp (Sorensen *et al.* 1988, Lim & Sorensen, unpublished data), this treatment is impractical because PGF2α is metabolized very quickly (i.e. 1-2 hours) so pheromonal activity is short lived. To overcome the limitations of injecting PGF2α, we have developed the use of osmotic drug pumps that can elicit the release of PGF2α into the body of carp for 1-3 weeks (exact duration depends on water temperature). Implanted fish can then be placed into traps or other locations and used as pheromonal 'bait.' Several aspects of using implants (dose, fish size and sex, etc.) have been optimised and are described below.

2.2. Conditions for using implants

Laboratory studies have now addressed all key parameters of how best to use implants that contain PGF2α to mimic natural pheromone production. These studies, detailed in a manuscript presently being prepared for peer-review (Lim & Sorensen, in preparation) are briefly summarized here. They have focused on determining the best dose (in terms of potency and cost) to implant and the effect of the gender of the implanted fish. Key results are as follows:

1. Laboratory behaviour tests of the potency of PGF2α-implanted carp odour versus the odour of naturally ovulated females

show that implanted carp odour is completely normal and should be fully effective in the field.

2. Laboratory tests using 1 kg carp and 2ML1 Alzet pumps show that the best dose to insert into these pumps is: 0.4 g PGF2α / kg fish. Doses higher than this killed implanted fish in 2-6 days. Lower doses produced odours that were less potent. Smaller and larger carp have not yet been tested.

3. The gender of the fish used for the implant seems not to matter – the odour of PGF2α-implanted males was as effective as that of implanted females in laboratory maze tests.

4. Implanted fish might be expected to survive for at least 2 weeks and release a full dose of the pheromone for the entire time.

5. The odour of an implanted fish held in 50 L of water for one hour remained attractive in laboratory maze tests when diluted 100,000 times. In field tests receptive male carp have been attracted from a distance of 75 meters in a still lake as evidenced by radio-tracking. These experiments were conducted in the middle of the spawning season when males were fully spermiated and known to be receptive (they were seen spawning). Laboratory studies have also shown that receptive females will follow males to the odour. No investigations have been undertaken with the effects of odour plume structure or to determine optimal trap design although we do know that carp are extremely 'trap shy.'

3. Making and deploying implants

3.1 Introduction

This section describes our recommendations for making PGF2α-implants for field use but are based on limited experience. We assume that the user is familiar with basic surgical techniques and the importance of sterility, and is working under animal ethics approval. Please note that PGF2α is active in humans and great care should be taken not to breathe or ingest it. Safety equipment including mask, gloves, and goggles must be worn. Familiarity with the Material Safety Data Sheet is assumed.

3.2 Materials needed

You will need several items to make and deploy implants. These are described below:

1) Carp (1–2 kg). These may be either sex but should be healthy.

2) Anaesthetic for carp. We recommend clove oil.

3) Model 2ML1 Alzet osmotic pumps (5.1 x 1.4 cm in length x diameter; Durect Corporation, CA, USA, http://www.alzet.com) (Figure 1). One 2ML1 can be implanted in a 1–2 kg fish and will pump for 414 hrs (~17 days) at 20°C or 431 hrs (~18 days) at 19°C (see instructions).

Figure 1. 2ML1 Alzet ostmotic pumps

4) Prostaglandin F2α (PGF2α). Prostaglandin F2α is a white crystal which can also be purchased as a salt from many sources (eg. Cayman Chemical, http://www.caymanchem.com/). Crystalline PGF2α should be stored in a dark, dry freezer (-20 °C). Many analogues exist and while untested, it is suspected they would also work. Prices vary enormously based on purity and amount purchased (research or medical grade is much more expensive than veterinary grade, and more expensive than an analogue). Most of our studies

have used medical grade PGF2α purchased wholesale from the manufacturer; however, a less expensive analogue was also used successfully (Dinoprost tromethyamine: Lutalyse, Pfizer Animal Health, http://www.lutalyse.com/). A 1 ml aliquot of Lutalyse solution contains 5 mg of dinoprost which requires appropriate concentration procedure to attain the optimal dose for the implant. This can be accomplished as follows:

a) Activate six C18 Sep-Pak cartridges (Waters, MA) by passing 5 ml of methanol and then 5 ml of distilled H_2O through them.
b) Dilute Lutalyse with distilled H_2O at a ratio of 1:50.
c) Pass aliquots of the diluted Lutalyse through activated C18 Sep-Pak cartridges at 1 Litre/hr.
d) Elute each C18 Sep-Pak with 5 ml MeOH (recovery rate should be 90%).
e) Collect the eluate (6 x 5 ml = 30 ml).
f) Evaporate the methanol using a nitrogen blower and add distilled H_2O (or 0.9% sterile saline) to make 2 ml solution for an implant.

5) Sterile surgical tools and sutures (the same tools as for implanting a radio-transmitter) (See section 4.1).

3.3 Loading PGF2α into implant pumps

Pumps must be filled prior to use. It is best to do this immediately before use to reduce chances of PGF2α breakdown or loss (not known although likely to be low). Dissolve stored PGF2α powder (or dinoprost) into clean distilled water or sterile saline to make a solution with a concentration of 200 mg / ml (this is about half of the solubility limit). The Alzet pumps prescribed take 2 ml and are suitable for 1–2 kg carp. If you have bigger carp, you may consider inserting multiple pumps. The solution should be sucked into the loading syringe (any 3 ml syringe works) and then injected into the pump using the blunt needle that comes with the syringe (Figure 2). It is essential that the pumps are completely filled. Detailed procedures for priming and filling a pump are available at www.alzet.com (see Products>Guide to Use>Filling & Priming), and also in the instruction sheet supplied with each box of pumps.

Figure 2. Loading PGF2α solution into a pump

4. Using implants

4.1 Introduction

Implantation is fairly simple and follows protocols similar to the implantation of radio-transmitters (see below). The biggest issue is designing an attraction protocol that makes sense. A few things to remember:

1) The implants will result in the production of a powerful female sex pheromone which means it will only attract fully mature and receptive males (although receptive [ovulated] females will school and follow males). Thus, this cue will only work immediately (i.e. a day or two] before, during, or just after spawning. We recommend using implants during and/ or just after spawning because it is the time that males may be most desperately searching for a few remaining ovulated females. The scheme should be carefully considered and focus on long-term reductions and/or censusing.

2) This sex pheromone is normally released via the urine, in a specific context, and as pulses. How, when, and where the implanted fish are deployed is likely to be very important. Unnatural settings or situations that are stressful for the implanted fish may not work. Also, the pheromone is probably best considered to be a short-to-moderate range synchronizer/arousal agent versus a long-range attractant. Natural dilution factors in the wild can be huge! Anything to promote or create sharp pheromone concentration gradients, such as placing implanted fish in a moving water area, will likely enhance activity.

3) Trapping/sampling protocol is essential. It probably is not reasonable to expect adult carp to force their way through tiny trap entrances just because a pheromone plume is in the general area. We recommend that you consider this cue as something that stimulates male searching to a general area that probably would be best captured with moderate sized gear such as large traps or pop-nets. Judas fish may also be used to aid location of fish.

4) Implanted fish may be toxic. Sacrifice them after the experiment and dispose of properly. (Being fatty acids, PGFs will break down rapidly but you do not want anyone eating them beforehand!)

4.1 Implantation

The implantation procedure should take about 5 minutes (Figure 3). Carp should first be lightly anesthetized using a technique you understand (we use clove oil). Once anaesthetized, you should then pick a location on the fish for the implant behind and above the pelvic fins. Remove scales from this area using forceps and carefully make

a small (~3 cm) incision using a sterile scalpel. Next, insert a filled pump, delivery port first, into the body cavity. Close the wound with absorbable monofilament suture (4 stitches, PDS-II, Ethicon). Resuscitate the fish using clean water.

Figure 3. The surgical implantation process

4.2 Maintaining and using implanted fish

Following implantation, and recovery from anaesthesia, the carp may be used immediately but, if possible, we recommend holding them for a day or two in clean water to monitor their health and condition. They should start feeding within a day or so. We have yet to experience any problems with infection but you could treat appropriately. If a fish does die, we do not recommend retrieving and reusing the used pump simply because we have no experience with this. Implanted fish can be held singly or as small groups in traps and enclosures.

4.3 Special considerations and possible problems

While potent, it is hard to overemphasize how targeted sex pheromones are. Our laboratory studies show that spermiated male carp which have not been exposed to sexually active female carp or their preovulatory pheromone (which is released during the 12 hour period proceeding spawning; Stacey & Sorensen 2009) are approximately 10 times less likely to respond to the female spawning pheromone than those that have been exposed (Lim & Sorensen, unpublished results). Timing of application must be precise. Of course, this could be advantageous if you know when carp spawn but a disadvantage if you do not. If pheromones are to be administered while spawning is occurring, high doses should be employed because the artificial cue presumably must compete with the natural one. Prostaglandin F2α may be needed in large quantities so it would be wise to contact industrial chemists in advance of need. High purity is probably not critical. Finally, even spawning male carp are trap shy; it would be wise to consider schemes which use larger trapping schemes that carp cannot detect.

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